CUTICULAR WAXES OF DEVELOPING LEAVES AND FRUITS OF CITRUS AND BLUEBERRY: ULTRASTRUCTURE AND CHEMISTRY

By

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I dedicate this manuscript to my wife, Claire, whose constant love and support made it all possible.

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Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the Requirements
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CUTICULAR WAXES OF DEVELOPING LEAVES
AND FRUITS OF CITRUS AND BLUEBERRY:
ULTRASTRUCTURE AND CHEMISTRY

By

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June 1978

Chairman: R. H. Biggs
Co-Chairman: L. G. Albrigo
Major Department: Horticultural Science (Fruit Crops)

Leaf and fruit waxes of 'Pineapple' and navel orange (*Citrus sinensis* [L.] Osbeck), 'Dancy' tangerine (*C. reticulata* Blanco), 'Eureka' lemon (*C. limon* [L.] Burm. f) (leaves only) and 'Bluegem' blueberry (*Vaccinium ashei* Reade) were studied between March and December 1977. Leaf wax concentrations were initially high, declined rapidly in March-April and increased to a maxima in June after which levels declined slightly in citrus and by 50% for blueberry leaves. June maxima values ranged from 26 $\mu g/cm^2$ for 'Pineapple' orange leaf wax to 40 $\mu g/cm^2$ for 'Dancy' tangerine leaf wax. Blueberry leaf wax was 135 $\mu g/cm^2$ in May. Citrus fruit wax concentrations generally declined in May-June and then increased through to November. Values ranged from 48 $\mu g/cm^2$ for mature 'Dancy' tangerine to 93 $\mu g/cm^2$ for mature navel orange and 295 $\mu g/cm^2$ for mature blueberry fruits. Intracuticular wax content ranged from 22 $\mu g/cm^2$ in blueberry leaves to 58 $\mu g/cm^2$ in navel orange fruits.

The ultrastructure of citrus leaf and fruit surface waxes developed similarly. The wax layer of immature leaves and fruits was initially

amorphous through which small protrusions and isolated regions of upright platelets emerged. All surfaces eventually cracked and uplifted to form large, flat irregular plates. Both fruit and leaf surfaces of blueberry developed extensive rodlet structures. The rodlets degraded rapidly on leaf surfaces, the wax on which became progressively more amorphous.

Secondary alcohols dominated citrus leaf waxes in March-April but quickly decreased or were diluted to trace levels. Primary alcohols were, overall, the major citrus leaf wax fractions, with paraffins being second in importance. Aldehydes and fatty acids were dominant in citrus fruit waxes. β-Diketones were the major fraction of blueberry leaf and fruit waxes. The loss of blueberry leaf wax after June was associated with the loss of almost all β-diketone. This loss was partially offset by a large increase in triterpenoids. Proportions of individual wax constituents changed constantly throughout the sampling period of all cultivars. Intracuticular waxes of all cultivars were mainly fatty acids.

A wick-feed technique was developed which permitted the extrusion and deposition of waxes on isolated citrus cuticles. Both citrus and blueberry waxes when reproduced were ultrastructurally similar to their natural forms. The rodlet waxes of blueberry were shown due to β-diketone, which transformed the latter to a rodlet structure when added to citrus waxes. The small platelets of citrus waxes were shown to be principally primary alcohols from immature leaves. No platelet structure was produced with primary alcohols from mature leaves. Wax ultrastructure, as developed by wick-feed, was shown to be influenced by relative humidity and air velocity.

Relationships between wax ultrastructure and chemistry in developing leaves and fruits are discussed.

INTRODUCTION

The plant cuticle, defined as the non-cellular membrane which covers epidermal cells, has received considerable attention among plant biologists in recent years. This is a result of improved techniques, particularly with electron microscopy and the recognition of the role of the cuticle in the plant's adaptation to stress and response to chemicals in agriculture.

Much of the previous work has been on mature cuticles with little emphasis on developmental changes, both morphologically and chemically. While some studies have investigated epicuticular wax development morphologically [2, 8, 38, 66, 84, 94, 142, 143, 164], chemically [29, 58, 65, 67, 72, 81, 180, 181, 166, 170, 186] and quantitatively [2, 4, 16, 50, 58, 157], apparently no studies have been undertaken to link these together.

Manipulation for increased plant productivity is a basic theme for most agricultural research programs. Epicuticular wax production and chemistry has been modified by the use of chemicals [39, 40, 45, 46, 51, 93, 112, 165, 185] primarily to influence the deposition and absorption of herbicides and pesticides. Structure of the cuticle and morphology and chemistry of cuticular waxes in relation to plant water loss [7, 8, 57, 137, 150, 151] and foliar uptake of nutrients and growth regulators [21, 37, 49, 75, 84, 108, 118, 132, 133, 144, 147, 152, 153, 169, 182, 189] has been investigated. But, apparently no studies have been

initiated to modify the chemistry and ultrastructure of surface waxes with the specific intent of improving the plant's adaptation to water stress. For this to be effective there need to be studies combining the developmental sequence of plant cuticle and quantitative, chemical and ultrastructural changes of cuticular wax and their response to environmental factors.

Development of epicuticular wax ultrastructure [2, 4, 42] and aspects of wax chemistry [15, 16, 121, 122, 123, 126, 127, 128, 129, 130, 131] in citrus have shown changes which may relate to alterations in the water relations of some species. The aims of this study were to examine wax chemical and ultrastructural aspects more closely in four citrus and one blueberry cultivar. Aims were fourfold: first, quantitative changes of leaf and fruit epicuticular waxes from budburst to maturity were to be determined. Secondly, these quantitative changes were to be related to ultrastructural changes as observed by scanning electron microscopy. The third aspect involved chemical fractionation of these waxes such that quantitative changes in the wax fractions could be correlated with total wax and ultrastructural changes. The fourth objective was to study more closely the relationships between wax chemistry and ultrastructure using a model system. The last would include a study of some environmental factors that may influence wax ultrastructure and attempts to change this by manipulating wax chemistry.

LITERATURE REVIEW:
THE NATURE AND FUNCTION OF PLANT EPICUTICULAR WAXES

Introduction

Plant cuticles are "protective skins." They are evident on the aerial parts of plants and since the times of de Bary, in the late nineteenth century, there has not been complete agreement amongst botanists regarding a precise definition. Martin and Juniper [117] defined the cuticular membrane as a

> multi-layered structure usually separated from the epidermal wall by a layer of pectin . . . This comprises an inner cuticular layer made up of cutin and cellulose, and an outer layer, the cuticle proper, made up entirely or mainly of cutin. (p. 4)

Waxy materials are embedded within and over the surface of the cuticle. Epicuticular wax is often referred to as the wax bloom. This may be prominent due to the reflection and scattering of light by wax deposits having dimensions similar to the wavelength of light. However, the bloom is not indicative of the amount of wax as some waxes are deposited as non-reflective plates or layers.

The "metacrase theory" expounded by de Saussure and others promoted controversy about the origins of the cuticle and epicuticular wax [117]. Cuticle was believed to be formed *in situ* by a process of modification of cellulosic constituents of the epidermal cell wall. It was first thought that wax was secreted to the surface in a volatile solvent or that it was formed from epidermal cell substances. In 1871

de Bary postulated the presence of canals and active wax secretion. The consensus by the mid-1920s was that waxes were formed in the cellular tissues and passed through micro passages to the surface where they hardened. Martens emphasized in 1933 the role played by secretion in formation of the cuticle and this led finally to the lapse of the "metacrase theory" [117].

The cuticle is a structural unit of the plant and holds cellular tissues compact and firm. It is a boundary between the plant and its environment, functioning as a barrier to water loss and leaching of cellular components. It also serves to protect the plant from injury, influences the deposition and behavior of chemicals and acts as a barrier to pathogens.

Wax Deposition

The method by which wax passes through the cuticle has been, and still remains, a matter of controversy. Mueller, Carr and Loomis [120] considered the early hypotheses of de Bary in 1871 and Dous in 1927, both of whom postulated that wax extruded through the cuticle, possibly through pores. Their own studies revealed pits in the leaves of and *Brassica* species, but wax removal showed that they had insufficient depth. Albrigo [2] observed small structures on immature 'Valencia' orange leaves not evident on mature surfaces. They were smaller than stomata and were plugged or coated with wax. The presence of pores 6-10 nm in diameter was shown in the cuticles of the leaves of *Trifolium repens*, *Brassica oleracea*, *Poa colensoi* and apple fruits; and in the leaves of *Eucalyptus urnigera* [60, 61, 62]. Pores were also observed in surface replicas of other plants including pea and wheat.

Scott et al. [160, 162] observed minute radial canals through the cuticle by irrigating sections of *Citrus sinensis* fruit and *Cercidium* seed with IKI-H_2SO_4. However, they found no evidence of canals in *Allium cepa* when the same techniques were used [161]. Fisher and Bayer [44] observed distinct channels through the cuticle of *Plantago major*. They were smaller than those proposed by Hall [61] having a diameter of about 2.5 nm but their presence did support Hall's hypothesis that wax precursors move via definite channels.

Pores or channels are by no means ubiquitous. They have not been found in *Brassica* or *Musa* species [149], *Eragrostis curvula* [109], *Phormium tenax* [86], *Pisum sativum* [93] or a range of other species [14]. Observations by Hallam [64] provide support for the view that wax lamellae occur within the cuticle itself. He suggested that these lamellae were wax precursors and could be indicative of a pathway through the cuticle. Hallam [64] also considered the pathways as anastomosing channels rather than a large number of pores between the epidermis and the exterior. Anastomosing channels were also suggested to be in *Apium* species by Chafe and Wardrop [26] and they equated these with those described by Hallam [64]. Hoch [74], however, considered these to be micro fibrils rather than wax pathways. This question is, therefore, still unresolved.

Methods of wax extrusion need to be considered in order to resolve more fully the question of how wax reaches the surface. Mueller et al. [120] concluded that wax was extruded as a softened paste under pressure and that this extrusion may have been through an apparently intact cell wall and cuticle. Schieferstein and Loomis [149] followed this concept and suggested that extrusion as a paste stopped when the leaf was more

mature due to a blocking of the wall and cuticle or both by a final hardening of the primary cuticle. A similar concept was proposed by Davis [38] who considered extrusion to be through pores which were dynamic and did not exist beyond a critical stage of growth. The structures observed by Albrigo [2] in immature but not mature citrus leaves support this theory. Hall [61] suggested that wax exuded almost certainly in liquid form and stresses in the cuticular membrane influenced the orientation of the pores and subsequent wax morphology. Baker's [10] studies with *Brassica* supported the view that wax was carried through the cuticle in a solvent. Extrusion as thick paste or through pores would not explain the rapid formation of different wax configurations on top of existing structures. The fact that some sites favored tube waxes while others favored plate waxes suggested to Baker there were localized areas with different rates of wax exudation or at different solute concentrations. He suggested the solvent may be volatile short chain aldehydes, ketones, alcohols and sulphur compounds. Insistence on the pore theory was maintained by von Wettstein-Knowles [178] who stated that wax polymerized on contact with air subsequent to extrusion through pores. Fusion of wax from adjacent pores prior to the completion of polymerization and the effect of continuous extrusion of new wax, variation in chemical composition and arrangement and densities of the pores were all considered factors explaining variations in wax morphology. This theory relies upon the existance of pores. However, variation in structure has been explained without reliance on a pore theory. Waxes have been recrystallized *in vitro* to produce forms almost identical to those occurring naturally and the relationship between chemistry and fine structure has been demonstrated by several

workers [66, 67, 169, 180]. Jeffree [88] and Jeffree et al. [89] convincingly supported this relationship. They used porous ceramic discs and demonstrated that extrusion through the pores played no part in the development of crystalline deposits. In a study on n-alkanes, Birdwell and Jessen [20] indicated that crystal habit depended on temperature and rate of crystallization, and this supports Baker's [10] view that wax structure is largely a consequence of environment. He showed large changes in morphology with only a slight change in chemical composition. Proposed pathways for wax transport should not be confused with those proposed for the movement of polar and apolar substances through the cuticle. Existence of specific pathways for the passage of polar compounds was proposed [31, 32, 144, 167] but never established experimentally [153]. Detection and study of ectodesmata [49, 158] led to new concepts of foliar penetration [49]. Ectodesmata were originally considered to be protoplasmic strands which penetrated the outer epidermal cell wall and terminated at the cuticle [158, 159]. Franke [48, 49] demonstrated, however, that ectodesmata were not protoplasmic strands but well defined structures in the epidermal cell walls rich in reducing substances. Their role as polar pathways in foliar absorption wax examined by Schonherr and Bukovac [154] who demonstrated sites in isolated cuticles which were preferentially permeable to mercuric chloride. They showed the ectodesmata, viewed as reduced mercuric chloride precipitates in the outer cell wall, were in fact artifacts, and that they could be identically reproduced in agar blocks underlying isolated cuticle [154]. Ectodesmata, therefore, appear only at sites of penetration in the cuticle and are a result of cuticular properties [118]. These sites normally occur over anticlinal walls and in

stomatal pores [153, 154, 189]. Removal or disorganization of surface wax revealed many more sites but in a random array [153, 154]. There needs to be a close examination of cuticular wax distribution on and within the cuticle in order to understand and explain this. Similar sites were demonstrated by Yamada et al. [189] using radioactive inorganic ions and urea.

Ectodesmata may represent sites for the absorption of polar compounds but, they do not relate to apolar substances. The possibility of multiple pathways has been proposed [49, 182] to account for movement of polar and apolar substances with the possibility of special pathways for cutinaceous material [49]. An additional pathway may be necessary for the transport of surface waxes. McFarlane and Berry [118] demonstrated cation penetration through isolated cuticles and suggested that polar pathways were pores lined with positively charged sites derived from proteins in the cutin matrix. More convincing evidence of polar channels was demonstrated by Schonherr [150, 151] who suggested that polar pores were due to clusters of COOH groups. Permeability of water through these polar pathways was pH dependent [150], while the permeability of non-polar substances was not pH dependent [152] and was generally positively correlated to the lipid solubility of the permeating species [23, 37, 147, 182]. Evidence suggests that apolar substances move within the lipid phase of the cutin matrix and not in the aqueous phase. Removal of epicuticular wax increases the permeability of both polar and apolar substances [21, 23, 132, 150, 151, 153] but no explanation has been offered to equate waxes with solubilities and alternate pathways of the permeating species.

Morphology of Cuticular Waxes

The nature of the cuticle surface is primarily a function of the cuticle itself rather than a reflection of the shape of underlying cells. Much of the surface detail is provided by those substances secreted onto the surfaces. This includes the fats and waxes as well as terpenoids, oils, carbohydrates and other substances. Waxes are usually responsible, however, and these were first seen by de Bary in 1871 as projections 10 to 20 nm long on *Strelitzia ovata* [117]. Four types of waxy coatings were proposed in which the wax particles were described as granules, rodlets, needles on one or more layers. It was not until 1967 that Amelunxen et al. [5] expanded de Bary's classification into six types, (1) granular coatings with a single layer of granular or spherical masses, e.g., *Rosa canina*; (2) rodlets and threads--straight, curved or coiled and which project from the epidermis, e.g., *Tulipa gesneriana*; (3) platelets and scales which lie flat on the cuticle or may be elevated and closely packed, e.g., *Dasylirion serratifolium*; (4) layers and crusts which may be smooth or show rodlike or other projections, e.g., *Vitis vinifera*; (5) aggregate coatings which consist of superimposed secretions of multiple structure, e.g., *Eucalyptus globulus* and (6) liquid or soft coatings consisting of droplets or irregular flat cakes, e.g., wax of *Malus domestica* fruits.

The diverse range of wax structures described in the literature makes any form of classification difficult and the system proposed by Amelunxen et al. [5] would now seem obsolete. This is largely due to the discovery of more wax types, particularly the wide range of intermediate structures that are difficult to categorize. The various

mutants of barley (*Hordeum vulgare*) have combinations of tubes, lobed plates and thin plates adpressed to the cuticle [177] and thick heaped layers of long needles have been described as being located on leaf sheaths, internodes and lemmas [89, 110, 178]. These needles were shown by negative staining to be tubes with a diameter of 100 nm and a core diameter of 30 nm. Also present were coiled ribbons which branch from the tubes and other structures were intermediate between tubes and coiled ribbons. Both wheat and barley had single and mutiple branched tubes and in some instances 70 nm ribbons topped by lobed plates. Leaf waxes of barley and wheat have plate structures [178] and these may be lobed in the case of wheat [125] and restricted to the adaxial leaf surface [169]. Other monocotyledons, which have tubular wax structures, are *Avena* species [19], *Zea mays* [117], *Musa* species [120] and *Agrostis curvula* [109]. A major difficulty of classification lies in the nomenclature of wax structural types. Similar structures on cereal organs have been called tubes [177], needles [110], filaments [18, 19] and ribbons [178]. Similarly, the terms "rods" and "rodlets" are widespread in the literature and require clarification.

Interpretation of wax structure, particularly the vertical tubular type is complicated due to the possibility of artifacts resulting from beam damage in scanning electron microscope studies and in the replication process with transmission electron microscopy. Parsons et al. [136] showed how beam damage could transpose vertical wax tubes to small knob-like structures. The papillae-like wax extrusions described by Fahn et al. [42] on grapefruit juice vesicles are suggestive of this, as are the "knobs" described by Baum and Hadland [19] for *Avena* species. Micrographs of Mueller et al. [120] lack a clarity of structure again

indicative of possible damage. Distortion in the replication procedure is evident in a micrograph of von Wettstein-Knowles [178] where the wax structure portrayed does not correspond with its shadow.

Plate waxes are as variable in form as tube waxes. They may develop from amorphous wax during maturation as is the case with citrus fruits [2] or they may develop directly as plates on other fruit or leaf surfaces as described below. Hallam and Chambers [67] classified plate structures of *Eucalyptus* species according to whether plate margins were entire, sinuate, crenate or digitate. Baum and Hadland [19] used only two criteria, entire or fringed, while von Wettstein-Knowles [177] referred to lobed plates. Again, lack of standardized descriptive nomenclature provides for confusion. Wax plates may lie flat, as in the case of barley [177], flat or overlapping, as in citrus [2] and grapes [27], closely packed with tubular protruberances, as in prune plums [7, 8] or dense and vertical, as in *Phormium tenax* [86]. Apples have been described as having plate wax but there is considerable variation among cultivars [154].

Many species of plants have a mixture of wax types, often upon the same organ. *Eucalyptus polyanthemos* has tubular wax on the leaf midrib and this is adjacent to a mixture of tubes and plates, while the outer region consists of plates only [67]. Amorphous waxes are also common and occur on citrus leaves [2], *Picea pungens* [69], nectarine [47], *Citrus limon*, peach, *Nicotiniana tobacum* and tomato [90].

Wax deposits are usually quantitatively greater on fruits than on leaves. Many fruits have in excess of 100 $\mu g/cm^2$, for example, *Cotoneaster cornubea* 240 $\mu g/cm^2$, banana 160 $\mu g/cm^2$, plums 120 $\mu g/cm^2$ [7] and 'Valencia' orange 178 $\mu g/cm^2$ [4]. Leaves generally have less

than 50 $\mu g/cm^2$, for example, citrus [4], potato, red beet, turnip, broad bean and tomato [117], while cabbage has 80 $\mu g/cm^2$ [117].

Cuticular Wax Development

The morphology and chemistry of wax on any plant species is subject to variation. A wide range of factors affect wax development and these are discussed in the following sections.

Temperature

Temperature affects total wax production, rate of wax production and wax composition. Daly [36] reported a slight positive correlation between leaf wax quantity of *Poa colensoi* and mean temperature. An increase in total wax with increasing temperature was also reported for *Prosopis juliflora* [83, 84]. Decreasing wax content with increasing temperature, however, has been reported for *Eucalyptus viminalis* in a temperature range of 18 to 33°C [117]. Similarly, Albrigo [4] reported reduced wax accumulation of Calamondin fruit at 27.5°C as compared with 21°C: and stored mandarin type fruit produced wax at 20°C but not at 30°C. Baker [10] compared wax production at 15°C, 21°C and 35°C on *Brassica oleracea*, with maximum production at 21°C.

The majority of reports have related quantitative wax production to temperature without paying much attention, if any, to the physiological significance of such phenomena. Temperature may be affecting availability of substrate, rate of reaction, biosynthetic pathway or the mobility of wax or wax precursors to the surface. Hull [83] first suggested that changes in wax chemistry may be involved and showed a direct correlation between melting point and day/night temperatures for the

surface wax of *Prosopis juliflora*. Changes in wax morphology with changes in temperature were reported for *Zizania aquatica* [71] and for *Brassica napus* [181]. The latter authors suggested that changes in wax structure were the result of changes in chemical content of wax or of changes in the rate of crystallization. Wilkinson and Kasperbauer [186] proposed a steady state hypothesis in which they state that wax chemistry and, therefore, wax morphology is a function of the environment. With each change, there is a new array of carbon-chain skeletons for synthesis. Giese [52] has shown that both light and temperature influence chain length and Baker [10] showed that temperature change altered the proportion of groups associated with specific pathways. Leaf waxes of *B. oleracea* at 15°C were principally rods, tubes and filaments composed of 51% alkane, 25% ketone, and 15% secondary alcohol. At 35°C the wax was mainly in the form of large dendritic plates composed of 30% alkane, 22% ketone and 13% secondary alcohol, with an increase in aldehyde of 14%.

Light Intensity

Earlier studies involving the effects of light on wax production were mainly concerned with total wax content or the morphology of the wax. They were unanimous in reporting reduced wax production and changes in morphology at reduced light intensities [9, 10, 40, 66, 94, 109, 116, 180, 181]. Chemical analyses of wax at differing light intensities revealed changes in chemical composition. Macey [111] showed that surface waxes of *B. oleracea* had more C_{29} compounds and fewer aldehydes and esters at higher light intensities.

Light dependence of wax development in plants is due to more than species differences according to Giese [52]. Using labelled precursors, Kolattukudy [97, 98] found that light had no effect on synthesis of hydrocarbons in *Brassica*, whereas Macey [111] showed that as light intensity increased in *Brassica* so did synthesis of hydrocarbons and secondary alcohols at the expense of aldehydes and esters. Formation of ketones was also light independent in the latter study. These studies do not agree with the work of Baker [10]. Unfortunately, none of the authors with the exception of Baker [10] has specified accurately the environmental conditions during the period of synthesis and direct comparisons are, therefore, impossible in view of the fact that Baker [10] showed that temperature, relative humidity and light all affected wax chemistry. Giese [52] showed that the amount of wax was 2.5 times greater in barley (*Hordeum vulgare*) seedlings grown at 14,000 lx., than that for dark grown seedlings. When the dark grown plants were transferred to light, the rate of wax production increased from 6 $\mu g/cm^2/24$ hr. (dark) to 46 $\mu g/cm^2/24$ hr. for the first 24 hours, then it settled down to 15 $\mu g/cm^2/24$ hr., a value equivalent to that of light grown seedlings. Alcohols (C_{26}), aldehydes (C_{20}) and free fatty acids (C_{22}, C_{24}, C_{26}) increased from 8 to 175 ng/cm^2, 2107 to 8152 ng/cm^2 and 204 to 902 ng/cm^2, respectively. Hydrocarbon (C_{21}, C_{23}, C_{25}) decreased at the same time from 16 to 3 ng/cm^2 and also free fatty acids (C_{34}, C_{36}, C_{38}) from 131 to 7 ng/cm^2 [52].

The synthesis of wax from palmitate or stearate that is produced in the chloroplast, and the influence of light upon this synthesis are complex. The elongation process responsible for paraffin synthesis is not directly coupled to photosynthetic reactions, therefore, Kolattukudy

[97] showed no inhibition during relatively short experimental periods (hours) in the conversion of acetate to paraffin in the dark. Macey [111] also reported that there was no effect of light on uptake of palmitate-1-^{14}C into wax but there was an increased synthesis of C_{29} compounds in light at the expense of aldehydes and esters. He suggested that light promotes formation of C_{29} compounds from C_{30} precursors and C_{30} is converted to C_{28} aldehyde and esters in the absence of light. Studies on biosynthesis have demonstrated that enzymes involved in epicuticular wax formation require substrates of specific chain length [22, 179]. Giese [52], in explaining changes in paraffin chain length at different light intensities, suggests there are at least two sets of enzymes with chain length specificity and these are differentially sensitive to light. One can, therefore, speculate on chain length specificity of enzymes for all of the wax classes and variations in wax chemistry at varying light intensity can be more easily rationalized.

Photoperiod

Relatively little has been done to elucidate the effects of photoperiod on plant waxes. That which has been done, alludes mainly to the overall effect and there has been no attempt to explain the effects at the cellular level.

Hawthorn and Stewart [71] observed little difference in the wax structures at the ultrastructural level of *Zizania aquatica* plant growth under continuous light and with shorter photoperiods. Wilkinson [183] studies the response of sicklepod (*Cassia obtusifolia*) to photoperiod as related to fatty acid synthesis. Total fatty acid content as well as the unsaturated and branched chain fatty acid was at a maximum

at 14 hours photoperiod. The odd-carbon numbered fatty acid content was highest at 16 hours, however, while the even-numbered chains were maximal at 14 hours. Wilkinson [183] observed that total fatty acid content of the waxes declined with age and it is, therefore, possible photoperiodic responses may be affecting the rate of physiological ageing and changes in wax chemistry may be a reflection of this. Photoperiod also affected alkane content of sicklepod [184] and in *Nicotiana tobacum* photoperiod and temperature affected alkane content [186]. The proportion of C_{31} and C_{33} alkanes dropped from 52% to 34% of total wax at 28°C when the photoperiod changed from 16 to 8 hours. Wilkinson [185] showed in a further study that photoperiod greatly influenced the quantity of each type of lipid wax and he attributed the changes as possibly being due to the differences in plant size at different photoperiods. Wilkinson and Kasperbauer [186] proposed a steady-state condition regarding alkanes and this could be extrapolated to include the other wax classes. A new array of carbon chain skeletons is available as the environmental conditions change; a new steady-state condition is established and the quality of the leaf wax reflects this.

Humidity

Few studies have investigated cuticular waxes in relation to humidity. Baker [10] studied normal and mutant forms of *B. oleracea* and concluded that the rate of radiant energy regulated the quantity of wax formed; the amount being greater at the high rate for each combination of temperature and humidity. Indications were that the effects of radiant energy on wax formation were influenced by changes in

relative humidity. A decrease in relative humidity had a stimulatory effect upon the production of wax and maximum deposits of wax occurred under condition of high radiant energy rate (80 Wm^{-2}) and low humidity. Thus, wax deposits at 21°C and 40% relative humidity were 76 $\mu g/cm^2$ compared with 33 $\mu g/cm^2$ at 21°C and 98% relative humidity. The smaller quantities of wax at high humidity were reflected in the restricted development of wax tubes and the replacement of dendritic plates by ribbon-like projections. Grout [55, 56] corroborated Baker's work when he cultured *B. oleracea* from meristem tissue and found there was reduced wax in the culture vessel at very high humidity. Wax levels could be increased by a hardening process in which relative humidity was decreased from 100% to normal ambient levels at a slow rate.

High humidity also depressed wax formation in stored citrus fruits [157]. The only possible report of the influence of humidity changes upon wax composition was by McNair [119]. He observed wax hydrocarbons, acids and alcohols from plants grown in the tropics had lower melting points and greater molecular weights than those in temperate zones. However, temperature and light must also have had an influence.

Plant Development

A study of 13 plant species by Kurtz [107] showed variation in wax yield with plant age. A slight increase in melting point with age in some species was correlated with a decrease in wax unsaturation. Acid components of wax declined in young plants and then slowly increased. Wax form varied with growth stage in wheat [169], *Aquilegia* species [143], *Brassica* spp. [94], *Citrus* spp. [2], *Phormium tenax* [86] and apples [11, 164]. Leaf wax deposits varying from 20 $\mu g/cm^2$ in oranges

to 31 $\mu g/cm^2$ in lemon were consistent in all except the most juvenile stages of growth [16]; whereas the amount of fruit wax varied considerably and showed a decline on a unit area basis in the mid-season period when fruit growth exceeded the rate of wax production [2, 11, 157].

The change from crystalline to amorphous wax, described for *Phormium tenax* [86] with increasing plant age, has been suggested as being due to a change in either wax chemistry or crystallization patterns. Reduction of soft wax on oranges with increasing age has been attributed to *in situ* atmospheric oxidation of wax [2]. A similar explanation was given by Kurtz [107] for changes in wax form as described previously. Increasing "greasiness" of apples in storage was attributed to an increase in the oil and fatty ester fractions [11].

More recent studies have indicated a marked change in wax chemistry with changes in plant age. In a study of several wheat varieties (*Triticum* ssp.), Tulloch [170] demonstrated a change from C_{31} hydrocarbons to C_{29} as maturity approached. Esters and β-diketones increased at the same time in relative proportions while alcohols decreased. Similar changes in chain length were reported for *Coffee arabica* [166]. Alkanes increased, the acids decreased and the alcohols remained relatively constant during leaf development. The ratio of C_{29}:C_{31} alkane changed from 1:1 66 days after germination to 1:2 at 112 days. The same changes in ratio were observed for C_{30}:C_{32} alcohols. Hudson and Karis [81] reported an increase in linolenic acid content and a decrease in palmitic acid content with maturity in kale, rye grass and fodder radish.

Changes in wax composition with plant age have been reported for many species. Most of the discussions have inferred that synthesis changes occur in the epidermal cells and new products then migrate to the surface as end products. Both Kurtz [107] and Albrigo [2] suggest that subsequent chemical changes occur by atmospheric oxidation and Albrigo [4] has demonstrated this *in vitro*. Wilkinson and Kasperbauer [186] have suggested a steady state hypothesis in which wax composition at any given time is a product of the precursors and biosynthetic pathways operable under specific environmental conditions. A more recent hypothesis suggests a steady state between the surface wax and the internal lipids and also among the different components of the wax [25]. Cassagne and Lessire [25] demonstrated reentry of certain wax components from the surface to the epidermal cells in studies with *Allium porrum* Thus, a cyclization of wax may be occurring in plants.

The Effect of Chemicals

Increasing the soil concentration of trichloroacetic acid (TCA) reduced total surface wax content and number of wax particles on the leaves of *Pisum* [93]. The melting point of the wax was lowered 4 to 5°C [39]. Macey [112] observed that TCA was more selective on the longer chain compounds although 10^{-4} M TCA inhibited synthesis of all wax components over an extended period of uptake. Similar effects were reported for cabbage [45, 46, 51] and *Pisum* spp. [105] using S-ethyldipropylthiocarbamate (EPTC). Wilkinson and Smith [187, 188] demonstrated that EPTC inhibited the incorporation of acetate-2-^{14}C into lipids in spinach chloroplasts and that the effects at 10^{-5} M could be reversed by the application of 1,8-naphthalic anhydride (NA)

and N,N-diallyl-2,2-dichloroacetamide (R-25788). Yet another herbicide S-(2,3-dichloro-allyl)di-isopropylthiocarbamate (Diallate) reduced wax synthesis quantitatively and was shown to influence wax composition by affecting the primary alcohols of *Pisum* [165] and the fatty acids, hydrocarbons and fatty alcohols of *Cassia obtusifolia* [185]. Wilkinson [185] also reported a Diallate-photoperiod interaction in which each lipid class responded differently to various combinations of the treatments.

Dithioerythritol and mercaptoethanol specifically inhibited alkane biosynthesis while wax ester and fatty aldehyde fractions increased [22]. Inhibition of C_{31} alkane by dithioerythritol and accumulation of C_{32} aldehyde suggested that inhibition was in terminal C_{31} synthesis.

Much of the research on the effects of chemicals upon surface waxes has been related to the study of herbicides which reduce wax levels. The possibility of manipulating wax levels to achieve an overall increase is suggested by the positive response with Benomyl and Difolatan upon citrus [4]. Albrigo [4] demonstrated that 1 ppm of ethylene in storage could promote wax production in 'Orlando' tangelos.

The Chemistry of Cuticular Waxes

The chemistry of cuticular waxes has been studied since the late nineteenth century. Much of the early work involved investigations on plants which produced waxes for commercial use, e.g., carnauba wax, candelilla wax, raphia wax and agave wax [117]. Earlier workers relied mainly on melting points and transition temperatures for identification of paraffins and alcohols, with little assurance that their substances were not mixtures. A major breakthrough resulted from the work of

Chibnall and Piper and colleagues [117]. They synthesized many long-chain compounds and determined their melting points, transition temperatures and crystal spacings. An extended range of reference compounds enabled them to analyze many plant waxes which led them to the conclusions that (a) the compounds found were saturated, (b) the paraffins, secondary alcohols and ketones had odd numbers of carbon atoms and (c) the primary alcohols and fatty acids had even numbers of carbon atoms. They considered all waxes to be the same general type containing odd carbon numbered n-paraffins from C_{25} to C_{37}, and even carbon numbered n-primary alcohols and n-fatty acids from C_{24} to C_{36}. However, the fact Chibnall and Piper used petroleum ether for wax extractions may have resulted in incomplete extraction of some wax components as more recent studies have tended to more polar solvents, such as chloroform [10, 16, 89, 151].

Alkanes

The wax hydrocarbon fraction represents a mixture of n-alkanes with 25 to 35 carbon atoms with a predominance of odd chains. As a percentage of total surface wax, there is a large variation in alkane content between and within species. Alkanes are a minor component in barley (*Hordeum* spp.) constituting 5 to 7% of stem and leaf wax and 12 to 17% of the wax on the culm [85], with n-tritriacontane being the dominant homologue in the leaf wax [176, 178] and n-hentriacontane the major one in the spike wax [85]. Giese [52] reported that the longer chain lengths C_{29} to C_{33} comprised 62% of the alkanes in barley leaves, particularly in older leaves, while short chain alkanes C_{16}, C_{17}, C_{18} comprised only 20%. Leaf wax of oats (*Avena* spp.) contained only 5%

alkanes, of which 78% was C_{29}, C_{31}, C_{33} homologues [173]. In comparison the wax of the wheat spike was composed of mostly alkanes, although in wheat leaves only 8 to 15% of the wax was alkane, principally n-nonacosane [125, 172, 174].

Low alkane contents were reported for the waxes of apple fruits (13%), apple leaves (16%) [103], sultana grapes (1%) [140], *Eucalyptus urnigera* (2.7%) and *Poa colensoi* (10%) [63]. n-Nonacosane was the major homologue in both the sultana and *Eucalyptus* waxes.

More than 50% of the leaf wax of *Pisum sativum* was n-hentriacontane [113] while alkanes comprised over 30% of total wax in *Brassica oleracea*, the prinicpal homologue being n-nonacosane [63, 73, 139]. Schulman and Monselise [157] reported that alkane content of 'Shamouti' orange peel wax was 11.4%. This was much lower than 40% for 'Valencia' oranges reported by Baker et al. [16], who also mentioned that the alkane content of lemon fruit wax was 23% as compared to 60% for lemon leaf wax and 43% for orange leaf wax. C_{31} and C_{33} paraffins were predominant in the citrus leaf waxes while C_{29} and C_{31} were predominant in the peel waxes.

Linear long chain hydrocarbons with odd carbon numbers predominate in most of the waxes studied to date. Variations do occur, however, and Herbin and Robins [72] showed that the dominance of odd- over even-carbon numbered alkanes decreased as the proportion of alkane in the wax decreased. Thus, C_{26}, C_{28} and C_{31} were predominant in *Solandra grandiflora*. In the juice sacs and seeds of 'Duncan' grapefruit, 44% and 53%, respectively, of the alkane were linear chains of length C_{25}. The remainder were iso- and anteiso-alkanes [124]. Only the peel wax

and leaf wax of the grapefruit had over 98% of the alkane in the linear form, which was n-hentriacontane.

Esters

Wax esters are generally made up of n-alkanoic acids and n-alkan-1-ols mainly with even numbers of carbon atoms, usually in the range of C_{12} to C_{32}. Free fatty acids and fatty alcohols usually correspond in structure to those of wax esters.

Esters were present in culm and leaf blade waxes of wheat and comprised from 9 to 25% of the wax. They were mostly long chain, even-carbon numbered (C_{44} to C_{56}) monoenes [125, 172, 174]. Esters (C_{44} to C_{52}) were also present but in lesser proportions, < 12%, in the leaf wax of oats [173], sultana grapes [140] and *Eucalyptus urnigera* [63]. Esters comprised 53% of the wax in *Portulaca oleracea* [171] and Schulman and Monselise [157] reported esters to be the major component (66%) of 'Shamouti' orange peel wax. In contrast, Baker et al. [16] found no evidence of esters in peel wax of four citrus cultivars. The discrepancy may be due to varietal differences.

Aldehydes

Relatively few species of plants have been recorded as containing aldehydes in their cuticular waxes. They occur in barley and wheat leaf waxes in minor proportions, the principal homologue being hexacosanal [52, 125, 178]. Approximately 12% of the surface wax of mature sultana grapes are aldehydes, the range of chain lengths being C_{24} to C_{28} with hexacosanal predominant [140]. Stem wax of sugarcane may contain up to 50% aldehydes [106]. Although Schulman and Monselise

[157] did not report any aldehyde in 'Shamouti' orange wax but Baker et al. [16] found them as a major component of lemon fruit wax, 43%, and orange fruit wax, 28%. In contrast, leaf waxes of lemon and orange contained only 0.3% and 0.1% aldehydes, respectively. The major homologues were C_{26} (lemon leaf), C_{24} (lemon fruit), C_{30}, C_{32} (orange leaf) and C_{26}, C_{28} (orange fruit). Both Baker et al. [16] and Schulman and Monselise [157] used similar methods for wax extraction and analysis and differences in aldehydes reported appear, therefore, to be varietal.

Alcohols

The major homologues of primary alcohols are generally of the same chain length as the aldehydes with C_{26} and C_{28} being dominant. Hexasosanol comprised 72% of barley leaf wax [173] and 40% of sultana grape wax [174] and sultana leaf wax [140]. The primary alcohols of citrus waxes comprised 37% of orange and lemon leaf wax (C_{26} to C_{32}) and 12 to 15% of orange and lemon fruit wax (C_{24} to C_{30}) [16]. Schulman and Monselise [157] reported that alcohols and ketones were only 2% of total 'Shamouti' orange wax.

Fatty Acids

Fatty acids occur with a wide range of chain lengths in most of the species which have been studied. The range for most plants is C_{16} to C_{34} for both ester and free fatty acids. With the exception of wheat (C_{16}) [174], the free fatty acids of the waxes of cereal leaves are in the C_{22} to C_{34} range, while ester fatty acids occur in the C_{16} to C_{22} range. Total fatty acid content of cereal leaf waxes was low at 2 to 3% [52, 171, 172, 174, 178]. The wax of apple fruits contained 7%

free fatty acids and up to 35% ursolic acid, a triterpenoid [163]. Oleic and linoleic acids comprise 77% of the fatty acids of 'Granny Smith' apple wax. Oleanolic acid, a triterpenoid, was a major component of sultana wax, 22%, while free and ester fatty acids combined comprised only 16%. The major free acid was n-hexacosanoic acid and the major ester-acid was n-eicosanoic acid [140]. n-Octacosanoic acid was the major homologue of fatty acids of sugar cane [106] and of coffee which also contained n-triacontanoic acid and n-dotriacontanoic acid [166]. n-Dotriacontanoic acid was also the major homologue of citrus leaf wax fatty acids which comprised 1.6% and 19.8% of lemon and orange leaf wax, respectively [16]. In both cultivars, fatty acids comrpised approximately 19% of total fruit waxes. The major lemon fruit wax homologue was n-triacontanoic acid and n-octacosanoic acid was the major homologue in orange peel wax [16]. Intracuticular waxes of citrus were principally fatty acids, 68 to 97%, with hexadecanoic acid, 44 to 78%, and octadecanoic acid, 11 to 39%, present as the major components [15].

β-Diketones

Long chain β-diketones were first reported as natural products by Horn and Lamberton [80]. They were shown to be major components, up to 50% or more, in leaf and stem waxes from a number of *Eucalyptus* and *Acacia* species, *Festuca glauca* and *Dianthus caryophyllus*. β-Diketones are readily characterized and estimated spectroscopically and can be separated from waxes as insoluble copper complexes. The most common β-diketone in the plants examined was n-triatriacontane-16,18-dione [80]. The principal β-diketone in many other plant species is n-hentriacontane-14,16-dione which is largely responsible for the tube

waxes [90, 177, 178]. It was the major component of glaucous barley lines [85, 177] and comprised up to 63% of internode wax [178]. It also comprised 6 to 10% of 'Little Club' wheat wax [174], 36% of 'Durum' wheat wax [172] and up to 55% of the wax on the sheath of wheat flag leaf [172]. This compared with 3% of the culm wax and 0% of leaf blade wax [125]. The same β-diketone comprised only 5.5% of the leaf wax of oats [173]. Four β-diketones were identified from the leaf wax of *Rhododendron* species [41]. n-Nonacosane-8,10-dione was identified in 22 species, n-nonacosane-12,14-dione in 1 specie, n-hentriacontane-14,16-dione in 5 species and n-hentriacontane-10,12-dione in 4 species [41].

Biosynthesis

Two major systems are responsible for wax biosynthesis. Fatty acid synthesis produces palmitic acid (C_{16}) and stearic acid (C_{18}) as end products. These are precursors of waxes which are synthesized by an elongation-decarboxylation system [89].

Fatty Acid Biosynthesis

Fatty acid biosynthesis was recently reviewed by Harwood [70] who discussed the major pathways, and by Stumpf [168] who examined in detail, the chloroplast, the site of synthesis.

Different plants have many features of fatty acid metabolism in common during germination and growth. These include the incorporation of acetate into saturated acids and synthesis of palmitate and oleate in isolated chloroplasts. Acetyl CoA, the most effective precursor of fatty acids, is formed from acetate by acetate thiokinase [70].

Malonyl CoA, the key intermediate in fatty acid biosynthesis, is formed from acetyl CoA by carboxylation and the synthesis of saturated fatty acids *de novo* is catalyzed by fatty acid synthetase. A central component of the reaction is acyl carrier protein which combines with acetyl and malonyl CoA during the synthesis reactions. Synthesis ends when palmitic acid has been synthesized. The mechanism of termination, however, has not been established. It is now considered that palmitate should be regarded as the end product of higher plant fatty acid synthetase reactions. Labelling studies have frequently confirmed that palmitate is made *de novo* whereas stearate is made by elongation [70, 115], in which malonyl CoA is added to a C_{16} precursor. Harwood [70] observed that evidence from a number of different types of experiments suggests sequential desaturation: thus--stearate → oleate → linoleate → linolenate.

The stroma phase of the chloroplast contains all of the enzymes responsible for *de novo* synthesis of fatty acids. Lamellar membranes are the site of O_2 evolution and generation of ATP and NADPH, essential components for fatty acid synthesis and desaturation. In contrast to yeast and animal systems, the *de novo* system in chloroplasts consists of soluble enzymes [168], i.e., they do not form a pellet with prolonged centrifugation at 10,000 g.

Although the products of acetate incorporation consists of C_{16} and C_{18} fatty acids, Stumpf [168] considered that a separate elongation system, other than the *de novo* system was operating in chloroplasts as well as in cytosolic systems. It was concluded that, in spinach chloroplasts, the complete machinery for *de novo* synthesis of 16:0 acid is present and the elongation to 18:0 ACP with subsequent desaturation to 18:1 ACP takes place [168].

Biosynthesis of Wax Components

The major distinction between internal lipids and cuticular lipids is that the former consist of C_{14} to C_{20} carbon chains, while the latter consist mainly of carbon chains longer than C_{20}. This discustion relates to the latter. In many young plant tissues, a variety of exogenous labelled fatty acids (C_2 to C_{18}) are incorporated into very long chain acids, probably by chain elongation [101]. Kolattukudy [101] suggested that the various classes of elongated products are made by different elongating systems, but the various elongating enzymes have not, as yet, been isolated.

Fatty alcohols were first shown to be derived from fatty acids in young leaves of *B. oleracea* [100]. The fatty acid was reduced to aldehyde and thence to the alcohol with NADH and NADPH being the preferred reductants, respectively. It has been suggested that ATP might play a significant role in controlling the synthesis of fatty alcohols [101].

Wax esters can be synthesized by three possible mechanisms, direct esterification between a free acid and a fatty alcohol, acyl transfer from a CoA ester to the hydroxyl group of the fatty alcohol or acyl transfer from a phospholipid to the hydroxyl group of the fatty alcohol. All three of these possibilities can be demonstrated in cell-free preparations under appropriate experimental conditions [101].

Biosynthesis of hydrocarbons was originally proposed as a head-to-head condensation mechanism [30]. It now appears synthesis of long chain hydrocarbons is the result of an elongation-decarboxylation mechanism [98]. According to this hypothesis, palmitic acid, the usual end product of fatty acid synthetase, becomes the substrate for an

elongation-decarboxylation system which adds C_2 units from malonyl CoA until the chain length of the acid reaches C_{30} or C_{32}. The elongated product is then decarboxylated, releasing the hydrocarbon. Some of the elongated chains are released from this complex, presumably as CoA esters which, in turn, are incorporated into fatty aldehydes, polar lipids and wax esters [101]. Use of inhibitors and mutants has been valuable in elucidating this mechanism. Trichloroacetate [98, 103] and several thiocarbamates [102] as well as the mutation in the gl3 mutant of *B. oleracea* [114], appear to block the elongation process, while certain thiol compounds [22] and the genetic alternation in WSP (*P. sativum*) and gl4 (*B. oleracea*) mutants [113, 114] inhibit the terminal decarboxylation step.

Biosynthesis of ketones and secondary alcohols is proposed as the result of hydroxylation of an alkane at a specific carbon in the middle of the chain, generating the secondary alcohol, which in turn is oxidized to the corresponding ketone [101]. This hypothesis is in good agreement with the observation that the chain length distribution of the oxygenated derivatives usually corresponds to that of the alkanes.

Site of Synthesis

It is generally accepted that surface lipids are synthesized in epidermal cells. Evidence suggests that C_{16}, C_{18} fatty acids are synthesized within chloroplasts while paraffins are synthesized outside chloroplasts [99]. Deposition of paraffins on the leaf surface occurs very quickly from the time of incorporation of labelled acetate [96]. Kolattukudy [99] stated that paraffin was deposited on the cuticle as soon as it was formed. It is usually assumed that paraffin synthesis

occurs in the epidermal cells rather than within the cuticle itself. However, recent studies by Sargent [146] have suggested that cuticular lamellae are involved in wax formation and she proposed that the polar lipids (fatty acids) provide *in situ* precursors for the synthesis of cuticular wax. This would require the presence of proteins (enzymes) in the cuticular lamellae to catalyze the elongation-decarboxylation system and these were shown to occur by specific staining [146]. Intracuticular wax of *Citrus*, as previously discussed, is mainly C_{16}, C_{18} fatty acids. This contrasts with the leaf surface waxes which are predominantly hydrocarbons, 23 to 66%, primary alcohols, 6 to 38%, and long chain fatty acids, 2 to 20%, [16]. Baker and Procopiou [15] suggest that accumulation of C_{16},C_{18} fatty acids within the cuticle may be indicative of the *in vivo* polymerization of cutin from these acids. Cutin is mainly a polyester of C_{16} and C_{18} hydroxy and other acids [13]. Cassagne and Lessire [24, 25] reported that reentry of certain wax components from the cuticle surface to epidermal cells of *Allium porrum* was possible. They suggested a cyclization of wax components between the surface and epidermal cells. However, their assessment of internal lipids was based on a methanol extraction following removal of surface waxes with chloroform. Methanol would also remove the intracuticular lipids and it is, therefore, possible that the cyclization of wax was not between the surface and epidermal cells, but between the surface and the cuticle.

MATERIALS AND METHODS

Leaf and Fruit Samples

Leaf and fruit samples were collected between March and December 1977 from mature trees of 'Pineapple' and navel sweet orange (*Citrus sinensis* [L.] Osbeck), 'Dancy' tangerine (*C. reticulata* Blanco), 'Eureka' lemon (*C. limon* [L.] Burm. f.) and bushes of 'Bluegem' rabbit-eye blueberry (*Vaccinium ashei*. Reade).

Epicuticular Wax Extraction

Initially composite leaf samples numbered greater than 1000 leaves and fruit samples 500 to 1000 fruits. These were reduced to 100 to 200 leaves and 10 to 20 fruits at the final samplings. Leaves and fruits were totally immersed and agitated in 500 ml chloroform at 55 to 58°C for 50 sec. Several extractions were necessary for each sample as the perforated stainless steel container held only 20 to 30 fully expanded leaves and ultimately only one mature fruit. The chloroform-wax solution was filtered, reduced to dryness in a preweighed flask and oven dried overnight at 55°C. Leaf area was determined initially by correlating the weight of cut-out leaf images (Xerox) with known areas. Most leaf areas, however, were determined using a Lambda Model L1-3000 portable area meter. Total leaf area was calculated by doubling the measured area. Fruit surface was calculated by first measuring volume by displacement of water and, assuming the fruit to be spherical,

correcting this to surface area using the formula

$$\text{Surface Area} = 4\pi\left(\sqrt[3]{\frac{3V}{4\pi}}\right)^2$$

where V = fruit volume. Newly set fruit received 5 x 10 sec. dips in five beakers of chloroform (55 to 58°C) to determine whether wax yield was being influenced by extraction of intracuticular or intracellular material in the later stages of the extraction period.

All extracted waxes were made up as 1% solution in chloroform and stored in a freezer.

Chromatography

Qualitative

Isolated waxes were examined for classes of constituents by thin-layer chromatography (TLC) on silica gel G using benzene-acetic acid (99:1) v/v mixture as developing solvent. Spots were detected by spraying with 0.05% aqueous Rhodamine-6G and observing under both long- and short-wave ultraviolet light. The spots were referenced using Rf values and chromatography with the internal standards listed in Table 1. Triterpenyl acetates and tritenpenoids were further identified using the Leiberman-Burchard test [79], infrared absorption and by TLC using benezene-chloroform (7:3) and chloroform-ethylacetate (1:1) as developing solvents [79]. Ultraviolet and infrared spectra were used to test for β-diketones.

Table 1. Standards used for identification of wax classes.

Standard	Class
Lignoceric acid	Fatty acid
Tricontanol	Primary alcohol
Hentriacontan-16-ol	Secondary alcohol
Hentriacontane-16-one	Ketone
Triacontylhexadecanoate	Ester
Hentriacontan-14,16-dione	β-Diketone
Docosane	Paraffin
Sugarcane wax	Aldehyde [106]

Preparative Layer Chromatography

Replicate wax samples of 10 to 14 mg were applied as bands to Gelman Instant Thin-Layer Chromatography (I.T.L.C.) sheets (Type SA) and fractionated into their constituent classes using the solvent system described for TLC. The bands were visualized by spraying with 0.05% aqueous Rhodamine 6G, thoroughly dried, cut into individual bands, eluted in boiling chloroform and filtered. The solvent was removed by evaporation and the classes determined gravimetrically. Each band sample was checked for purity using TLC.

Scanning Electron Microscopy (SEM)

Subsamples of leaves and fruits were taken prior to each wax extraction. Sections of 5 mm^2 were cut from the abaxial and adaxial

leaf surfaces and fruit surfaces. These were fastened to plastic petri dishes using a drop of citrus oil [1] and air dried in a desiccator. Individual leaf lengths and fruit diameters were recorded.

Air dried samples were cut into 2 to 3 mm squares and fastened to aluminum stubs using either silver conductive glue or "Avery" self adhesive paper tacks. The stubs were coated with approximately 100 Å of gold-palladium (60:40) on a "Technics" sputter coater and observed on a JEOL JSM-35 or a Novascan Scanning Electron Microscope operating at 15 to 25 kv and 100 μA current.

Soft Wax Determination

From each wax stock solution, 2 to 5 mls were taken and the solvent removed by evaporation. Oven-dry aliquots were weighed and refluxed with 50 ml of petroleum ether (30 to 60°C) for 1 hour. The wax was broken up with a spatula prior to refluxing. Solutions were cooled following refluxing to precipitate insoluble fractions and were filtered. The "soft" petroleum ether-soluble fraction was determined gravimetrically following evaporation of the solvent. Soft wax was determined as a percentage of initial wax weight.

The "soft" and "hard" (P.E. insoluble) wax fractions were examined by TLC.

Cutin and Intracuticular Waxes

Discs were cut from both dewaxed leaves and fruits at periodic intervals during the sampling periods. Discs were placed in a solution of 1 gm $ZnCl_2$ + 1.7 ml conc. HCl, using 4 to 5 ml per disc [77]. These were left for 24 to 48 hours with 2 or 3 agitations, filtered and

washed through a wire gauze grid. Cuticles were suspended in distilled water to float off additional debris, refiltered and resuspended in water. Three to four replications of from 100 to 300 discs each were taken per sample, oven dried at 55°C in weighing bottles and initial cuticle weight recorded. Several of each of the dried cuticles were stored for examination by SEM, while the bulk of each sample was extracted for intracuticular lipids using the method described by Baker and Procopiou [15]. Cuticles were refluxed for 2-hour periods successively with 100 ml quantities of methanol, methanol-chloroform (1:1) and chloroform. The combined extracts were evaporated to dryness and then refluxed with 25 ml hexane for 15 minutes. The hexane solution was cooled, filtered and oven dried to determine the weight of intracuticular lipids expressed as $\mu g/cm^2$ cuticle. Cuticle discs were recovered, oven dried (55°C) in weighing bottles and stores for later use. Intracuticular lipids were examed by TLC.

Wax Extrusions and Manipulation Studies

A modified wick-feed method similar to that described by Jeffree [88] was used to recrystallize a range of epicuticular waxes under a variety of conditions. Vials (2 ml) with a neck diameter of 5 mm were used. A wick of tightly rolled glass microfiber paper (Whitman GF/C) was inserted into each vial. Wax solutions, 1.5 ml using chloroform as solvent, were injected into the bottom of the vials by inserting a syringe needle down the side of the wick. A cuticle disc, prepared as described above, was placed over the wick in the neck of each vial. A flanged brass nut held the cuticle while allowing solvent evaporation to proceed. Cuticle discs were mounted on stubs after all of the

solvent had evaporated using "Avery" self adhesive paper tacks and prepared for SEM as described previously.

Wax Load

Preliminary studies to determine the optimum wax:solvent ratio for depositing wax similar in quantity and appearance to that which occurred naturally, used four concentrations for each of the leaf waxes of 'Bluegem' blueberry, 'Pineapple' orange, 'Dancy' tangerine and navel orange. Concentrations were 1.1 mg, 0.55 mg, 0.28 mg and 0.09 mg, expressed as mg wax per 1.5 ml solvent.

Cuticle Type

Totally dewaxed citrus leaf cuticle discs from each of the cultivars were used. Some of the cuticles were placed on the wicks upside down to test for reverse flow and some were only surface dewaxed. Both adaxial and abaxial cuticles were used.

A Study of Some Environmental Influences Upon Wax Ultrastructure

Temperature, wind and relative humidity

Two replicates using the wick-feed method described were used for each of the following treatments:

(1) 21°C and air velocity 0Kmh^{-1}

(2) 21°C and air velocity 8Kmh^{-1} (15 cm below the nozzle of a hairdryer on cool setting)

(3) 35°C and air velocity 0Kmh^{-1}

(4) 21°C and low relative humidity (desiccator)

(5) 21°C and high relative humidity (saturated chamber).

Totally dewaxed cuticle discs from 'Pineapple' orange and 'Dancy' tangerine leaves were used. Wax solutions were made up to 0.44 mg/1.5 ml $CHCl_3$ from blueberry leaf wax extracted from immature leaves in March 1977, and 0.55 mg/1.5 ml $CHCl_3$ from 'Pineapple' orange leaf wax extracted from immature leaves in April 1977 and mature leaves in November 1977. When the solvent had evaporated, the dry cuticle discs were mounted for SEM.

Ultraviolet radiation

The wick-feed technique previously described was used to study the effects of UV-B irradiation on wax ultrastructure and chemistry. In a preliminary study, blueberry wax from leaves collected in March 1977 (0.44 mg/1.5 ml $CHCl_3$) was injected into two vials with tangerine leaf cuticle discs. Wax deposition was allowed to occur with the vials 10 cm below two Westinghouse FS-40 lamps shielded with 3 mil (.076 mm) cellulose acetate sheets (changed every 12 hours), which filtered out wavelengths shorter than 292 nm. Total flux was measured with a spectroradiometer (Optronics Model 741) at the beginning and end of the radiation period of 31 hours. Two other vials were set up similarly but without UV radiation. All cuticle discs were examined by SEM.

A subsequent study investigated the effects of 72 hours continuous UV-B radiation on blueberry and tangerine leaf waxes and components from immature leaves collected in March 1977. All other conditions remained as above. The following treatments using totally dewaxed tangerine leaf cuticles were replicated twice at ambient temperature and examined by SEM:

1. Blueberry leaf wax irradiated during extrusion and for an additional 72 hours at ambient temperature (21°C).

2. As in (1) but held at ambient temperature (21°C) for 72 hours without UV.

3. Blueberry leaf wax irradiated with UV light for 72 hours in a glass, open petri dish and then made up to 0.55 mg/1.5 ml $CHCl_3$ and injected into vials for extrusion.

4. As in (3) but without UV irradiation.

5. β-Diketone separated from blueberry wax by preparative TLC and treated as in (3).

6. As in (5) but without UV irradiation.

7. Tangerine leaf wax treated as in (3).

8. As in (7) but without UV irradiation.

The 3 mil cellulose acetate sheets were replaced each 12 hours to avoid excessive solarization. All of the waxes used in each study were examined by TLC.

A Study of the Influence of Wax Chemistry on Ultrastructure

Totally dewaxed 'Pineapple' leaf cuticle discs were used in conjunction with the wick-feed techniques. The following treatments were replicated twice at 35°C in still air, after which the cuticles were examined by SEM:

1. Blueberry fruit wax collected in April (0.55 mg/1.5 ml $CHCl_3$).

2. 'Pineapple' orange leaf wax collected in March (0.55 mg/1.5 ml $CHCl_3$).

3. Blueberry fruit wax constituent classes reassembled following separation by preparative TLC (0.55 mg/1.5 ml $CHCl_3$).

4. Blueberry fruit wax constituent classes reassembled as in (3) but without the β-diketone component (45% of total berry wax).

5. 'Pineapple' orange leaf wax constituent classes reassembled following separation by preparative TLC (0.55 mg/1.5 ml $CHCl_3$).

6. 'Pineapple' orange leaf wax plus the β-diketone removed from the blueberry fruit wax so that the β-diketone was 45% of the total citrus wax.

Leaf waxes collected from blueberry and all of the citrus cultivars, except lemon, sampled in March and November 1977 were compared using the wick-feed technique. Mature and immature 'Pineapple' fruit waxes were also compared. Totally dewaxed 'Pineapple' leaf cuticle discs were used and all wax concentrations were 0.55 mg/1.5 ml $CHCl_3$. Extrusion was carried out at 35°C in still air and the cuticles were examined by SEM.

Tangerine leaf waxes collected in March and November were fractionated using preparative TLC. The following constituents were eluted and made up to 1.1 mg/1.5 ml $CHCl_3$ and compared using the wick-feed through dewaxed tangerine cuticles:

(1) Secondary alcohol from March sample,

(2) Primary alcohol from March sample and

(3) Primary alcohol from November sample.

These were subsequently examined by SEM.

RESULTS AND DISCUSSION

Developmental Study

Scanning Electron Microscopy

Leaf surfaces

Changes in surface wax ultrastructure with leaf development are shown in Figures 1-8 for 'Pineapple' orange, Figures 9-16 for 'Dancy' tangerine, Figures 17-24 for navel orange, Figures 23-30 for spring flush 'Eureka' lemon and Figures 31-38 for 'Bluegem' rabbiteye blueberry.

Citrus. The most immature leaf samples of all the citrus cultivars and blueberry had smooth amorphous wax layers (Figures 1, 9, 17, 25, 31). This is not uncommon and has been reported for the immature leaves of 'Valencia' orange [2], *Phormium tenax* [86], *Aquilegia formosa* [143] and *Prunus domestica* [8].

As the leaves rapidly expanded, new wax began to protrude through the amorphous layer in a variety of conformations. The first visible wax structures on 'Pineapple' orange leaves were small, round protrusions irregularly oriented (Figures 2, 4, 5) or aligned vertical platelets (Figure 3). Vertical platelets were observed in the other citrus cultivars and were reported in 'Valencia' orange [50] as a result of polishing the surface. Hall [59] described similar structures, also

Figures 1-4. Wax development on the abaxial surface of 'Pineapple' orange leaves.

Fig. 1. Sampled March 24, leaf length 5.2 cm.

Fig. 2. Sampled April 8, leaf length 8.8 cm.

Fig. 3. Sampled April 8, leaf length 10.2 cm.

Fig. 4. Sampled June 28, leaf length 7.2 cm.

1
3.9μm
2
8.4μm
3
4.7μm
4
6.2μm

Figures 5-8. Wax development on the abaxial surface of 'Pineapple' orange leaves (Continued).

Fig. 5. Sampled June 28, leaf length 10.0 cm.

Figs. 6-7. Sampled December 15, leaf length 8.2 cm.

Fig. 8. Sampled December 15, leaf length 9.1 cm.

5
2ثانية
6
4ثانية
7
6ثانية
8
4ثانية

Figures 9-12. Wax development on the abaxial surface of 'Dancy' tangerine leaves.

Fig. 9. Sampled March 24, leaf length 2.1 cm.

Fig. 10. Sampled March 24, leaf length 4.0 cm.

Figs. 11-12. Sampled April 8, leaf length 8.0 cm.

9
3·4μm
10
1·4μm
11
5·4μm
12
0·8μm

Figures 13-16. Wax development on the abaxial surface of 'Dancy' tangerine leaves (continued).

Figs. 13-14. Sampled August 10, leaf length 8.0 cm.

Fig. 15. Sampled November 15, leaf length 7.9 cm.

Fig. 16. Sampled June 28, leaf length 6.7 cm.

13
8·5μm
14
8·8μm
15
6·6μm
16
8·3μm

Figures 17-20. Wax development on the abaxial surface of navel orange leaves.

Fig. 17. Sampled March 26, leaf length 4.7 cm.

Fig. 18. Sampled March 26, leaf length 5.8 cm.

Fig. 19. Sampled April 10, leaf length 6.5 cm.

Fig. 20. Sampled April 10, leaf length 8.2 cm.

17
8.9μm
18
3.5μm
19
4.2μm
20
10.8μm

Figures 21-24. Wax development on the abaxial surface of navel orange leaves (continued).

Fig. 21. Sampled May 15, leaf length 9.3 cm.

Fig. 22. Sampled October 6, leaf length 8.2 cm.

Fig. 23. Sampled August 21, leaf length 11.1 cm.

Fig. 24. Sampled October 6, leaf length 8.2 cm.

(21)
4.2μm
(22)
5.7μm
(23)
4.0μm
(24)
3.4μm

Figures 25-30. Wax development on the abaxial surface of spring flush 'Eureka' lemon leaves.

Fig. 25. Sampled April 9, leaf length 6.0 cm.

Fig. 26. Sampled April 9, leaf length 8.0 cm.

Fig. 27. Sampled June 28, leaf length 9.2 cm.

Fig. 28. Sampled August 10, leaf length 11.0 cm.

Fig. 29. Sampled September 27, leaf length 9.6 cm.

Fig. 30. Sampled December 15, leaf length 10.2 cm.

(25)
2.7μm
(26)
3.1μm
(27)
(28)
5.1μm
(29)
13μm
(30)

Figures 31-34. Surface wax development on 'Bluegem' blueberry leaves.

Figs. 31-32. Sampled March 11, leaf length 1.7 cm: abaxial.

Fig. 33. Sampled March 26, leaf length 4.1 cm: abaxial.

Fig. 34. Sampled March 26, leaf length 4.6 cm: adaxial.

(31)
7.3μm
(32)
0.7μm
(33)
3.9μm
(34)
3.3μm

Figures 35-38. Surface wax development on 'Bluegem' blueberry leaves (continued).

Fig. 35. Sampled April 9, leaf length 5.4 cm: abaxial.

Figs. 36-37. Sampled May 14, leaf length 6.0 cm: abaxial.

Fig. 38. Sampled November 21, leaf length 6.2 cm: abaxial.

35
5.3μm
36
6.7μm
37
8.3μm
38
3.3μm

a result of polishing, in apple wax. The relationship between the structures described here and abrasion or mechanical injury was not determined but the lack of any visible signs of injury suggests that the structures are a natural phenomenon associated with rapid leaf growth and wax development. The first visible wax structures on 'Dancy' tangerine leaves were small rounded protrusions and large flattened plates (Figures 10, 11) which were not firmly adhered to the underlying surface and were often observed to have lifted and rolled back (Figure 12). Wax protrusions on navel orange leaves were similar to those described for 'Pineapple' and 'Dancy' tangerine, but were more closely spaced, imparting a "pebbly" appearance to the surface (Figures 18, 19). Denser regions had a crusty mesh-like appearance (Figures 20, 21) which was often cracked and showed signs of breaking up. Wax protrusions on 'Eureka' lemon leaves were either aggregated or rounded and flattened to produce granular (Figure 25) or etched (Figure 26) surfaces, respectively. The small vertical platelets observed in Figure 26 are similar to those previously described on 'Pineapple' orange leaves but are less frequent and randomly dispersed. Emergent wax is crystalline in other plant species [7, 8, 18, 86, 143], in contrast to the amorphous forms observed in citrus.

Wax development subsequent to the changes described was similar for all of the citrus cultivars. In some areas of the leaves, wax production was quite prolific (Figures 4, 13) but such areas were often scattered between areas of lesser wax production. Two common features of wax ultrastructure were developed in all cultivars. The ridges or higher points of the leaf surface developed smooth wax which appears to have been deposited over the older wax and then flowed down each side

of the ridges to accumulate at the base and solidified (Figures 6, 14, 22, 28). The probable lack of temperatures high enough to melt the wax precludes melting as an explanation of this wax form. Cracking and lifting of the wax layers was common to all of the citrus cultivars. This was usually associated with the more mature leaves but was also observed on relatively immature 'Eureka' lemon (Figure 27) and navel orange (Figure 20) leaves. Uplifting of large areas of wax was best seen around the stomata (Figures 7, 15, 23, 28). The wax eventually broke into irregular plates which appeared to be loosely attached to the surface (Figures 8, 16, 24, 28). There appeared to have been a loss of the broken wax layer in most instances, possibly a result of wind, rain or abrasion. Albrigo [2] did not observe any uplifting or cracking of the leaf wax of 'Valencia' oranges. This is probably due to Albrigo's observations ceasing at full leaf expansion as Leece [108] examined more mature 'Valencia' orange leaves and did observe structures similar to those described here. The causes of uplifting, cracking and plate formation were not elucidated but may be a combination of factors including stresses (e.g., shrinkage) within the leaf, new wax accumulating underneath and changes in chemistry. There appears to be new wax development where plate waxes were removed in Figures 8 and 16. The implications of wax chemistry are discussed in a subsequent section.

Blueberry. Development of blueberry leaf wax contrasts strongly with that of citrus leaf wax. The initially amorphous wax layer (Figure 31) developed a rippled surface prior to the emergence of wax rodlets. These rodlets first appeared on the stomatal antechambers (Figures 31, 32). This has been observed in other species which produce

rodlet wax [142, 175] and may reflect advanced physiological maturity of cells around stomata or the greater number of chloroplasts usually associated with guard cells. Rodlets may be single, multiple and branched or both when extruded (Figure 32). They develop rapidly and cover the entire leaf surface (Figure 33). The degree of branching and interconnecting among the rodlets is seen in Figure 34, which also shows upright and flattened plate structures, the nature of which is not clear. The delicate nature of rodlets was demonstrated by the beam damage incurred in the SEM at 20kv. These structures could be viewed safely only at 15kv and for the thinner rodlets occasionally observed, 10kv. Amorphous waxes of citrus by contrast, were not affected at 20kv. Loss of leaf rodlet structure was observed as early as April (Figure 35) and this progressed until all of the rodlet structure was lost on senescent leaves (Figures 35-38). The sensitivity of the rodlets to beam damage in the SEM may reflect their susceptibility to weathering and this is considered to be the prime cause of the structural changes. Figures 35 and 36 show fusion of the rodlets and ultimate total loss of structure. A similar loss of rodlet structure was observed in *Picea pungens* [142] and this was attributed to weathering. Much of the blueberry rodlet structure had degraded by May with the exception of a secondary extrusion of new rodlet wax over the stomatal antechambers (Figure 37). Only amorphous wax remained by leaf senescence in November over which fungal mycelium had developed (Figure 38).

Fruit surfaces

Changes in surface wax ultrastructure with fruit development are shown in Figures 39-44 for 'Pineapple' orange, Figures 45-50 for 'Dancy'

tangerine, Figures 51-56 for navel orange and Figures 57-64 for 'Bluegem' blueberry.

Citrus. Wax of the immature fruit of each cultivar was mostly amorphous (Figures 39, 45, 51). 'Pineapple' orange fruit developed a thick and pebbly surface which often appeared layered (Figure 40), while emerging wax was mostly as small, rounded protrusions on 'Dancy' tangerine fruit (Figures 46, 47). The irregular protrusions observed on navel orange fruit are shown in Figure 52. Fruits of all three cultivars had areas of upright wax platelets (Figures 48, 53). These are more rounded with their edges more lobed than those found on leaves. They are similar to the plate structures described by Baum and Hadland [18, 19] for leaf waxes of *Avena* species. Navel orange fruit wax formed "crusts" in many areas and these were prone to lifting and cracking (Figure 54). Wax deposits varied in thickness and type on 'Pineapple' orange fruit. The thick deposits of amorphous wax shown in Figure 41 contrasted with the relatively thinner "pebbly" crust shown in Figure 42. Lifting and cracking was observed in all surface waxes as fruits approached maturity (Figures 43, 48, 55). The uplifted wax plates converged and buckled upwards as though they had compressed together on 'Pineapple' orange and navel orange (Figures 43, 44, 55). This is in contrast to the leaf wax plates which separated from each other. Plate wax development on 'Dancy' tangerine was much reduced (Figures 49, 50) and probably reflects lower wax levels than 'Pineapple' and navel orange fruits. Plate wax development was described for 'Valencia' orange fruit surfaces [2] but compression buckling as seen on 'Pineapple' and navel orange was not shown. It seems unlikely that

Figures 39-44. Surface wax development on 'Pineapple' orange fruit.

Fig. 39. Sampled May 12, diameter 1.8 cm.

Fig. 40. Sampled June 28, diameter 3.3 cm.

Fig. 41. Sampled September 27, diameter 6.9 cm.

Fig. 42. Sampled December 15, diameter 7.7 cm.

Figs. 43-44. Sampled November 15, diameter 7.1 cm.

(3E)
6.1μm
(4E)
5.4μm
(4F)
4.6μm
(4Z)
8.8μm
(4G)
5.3μm
(4H)
1.2μm

Figures 45-50. Surface wax development on 'Dancy' tangerine fruits.

Fig. 45. Sampled May 12, diameter 0.9 cm.

Figs. 46-47. Sampled June 28, diameter 3.2 cm.

Fig. 48. Sampled August 10, diameter 4.9 cm.

Figs. 49-50. Sampled September 27, diameter 6.5 cm.

(45)
6·4μm
(46)
2·8μm
(47)
7·5μm
(48)
6·5μm
(49)
2·8μm
(50)
7·9μm

Figures 51-56. Surface wax development on navel orange fruit.

Fig. 51. Sampled May 15, diameter 1.1 cm.

Fig. 52. Sampled July 10, diameter 5.0 cm.

Fig. 53. Sampled August 21, diameter 6.7 cm.

Fig. 54. Sampled October 6, diameter 8.9 cm.

Fig. 55. Sampled November 20, diameter 7.8 cm.

Fig. 56. Sampled December 14, diameter 8.0 cm.

(51)
(52)
(53)
(54)
(55)
(56)
2.4μm
4.9μm
2.4μm
1.6μm

Figures 57-60. Surface wax development on 'Bluegem' blueberry fruits.

Fig. 57. Sampled April 9, diameter 0.9 cm.

Figs. 58-59. Sampled April 24, diameter 1.0 cm.

Fig. 60. Sampled May 15, diameter 1.3 cm.

(57)
3.9μm
(58)
6.7μm
(59)
5.0μm
(60)
7.4μm

Figures 61-64. Surface wax development on 'Bluegem' blueberry fruits (continued).

Fig. 61. Sampled May 15, diameter 1.3 cm.

Figs. 62-64. Sampled June 5, diameter 1.6 cm.

(19)
1·3μm
(20)
4·0μm
(21)
2·7μm
(22)
3·7μm

plate buckling is a consequence of large wax deposits as Albrigo [2, 4] reported that 'Valencia' fruits had greater wax deposits than the other cultivars studied here. It does seem probable, however, that diurnal fruit volume changes in addition to expansion growth could create sufficient tensions to cause the wax to crack and would be more likely to result in buckling. The most mature fruit samples are represented in Figures 44, 50 and 56 and the breakup of the wax layer is quite apparent. Loss of the wax plates from the surface was most obvious in navel orange (Figure 56). Whether the waxes were actually lost from the surface or redistributed is not clear, but it is likely the loss was due to a combination of both factors. The underlying surface exposed as a result of plate wax removal in Figure 56 shows evidence of new wax production. This new wax may have contributed to the breaking and removal of the surface wax. It is significant that none of the fruit surfaces observed exhibited the smooth ridges and apparent wax flow common to all of the leaf surfaces. This may be a consequence of differences in wax chemistry as will be discussed in a subsequent section.

Citrus fruit have amorphous cuticular waxes which develop flattened plates as they mature. Other fruit with amorphous wax deposits and which develop similar plates include several apple cultivars [43, 45, 59, 164], pear [104] and grape [137]. Most other fruit surfaces have distinctive structural wax forms such as vertical platelets, granules or various crystalline forms [117]. The consequence of cracking and platelet formation in citrus fruit waxes was discussed by Albrigo [2, 3], who suggested that this may be responsible for dehydration and accelerated ageing.

Blueberry. Blueberry fruit wax development closely paralleled blueberry leaf wax development. Rodlets were observed on immature fruit (Figure 57) which had a prominent waxy bloom in the field. The fruit wax deposits were variable. Figures 58 and 59 represent samples of similar age and it can be seen that while rodlets are just developing in some areas, they are fully developed in others. Figures 60 and 61 show the sparseness of rodlets in some areas and the variability in rodlet structure. Thin rodlets appear to emerge directly from the surface while thick ones develop from mounds of amorphous wax (Figure 61). Degradation of rodlet structure, similar to that described for blueberry leaf waxes, occurred in the more mature fruit (Figure 62). The ultrastructure on ripe berries varied from the narrow rodlet structures observed in Figure 63 to the short, stubby rodlets and amorphous combinations of wax forms seen in Figure 64. Rodlet structure did not persist longer on fruits than leaves.

Quantitative Data

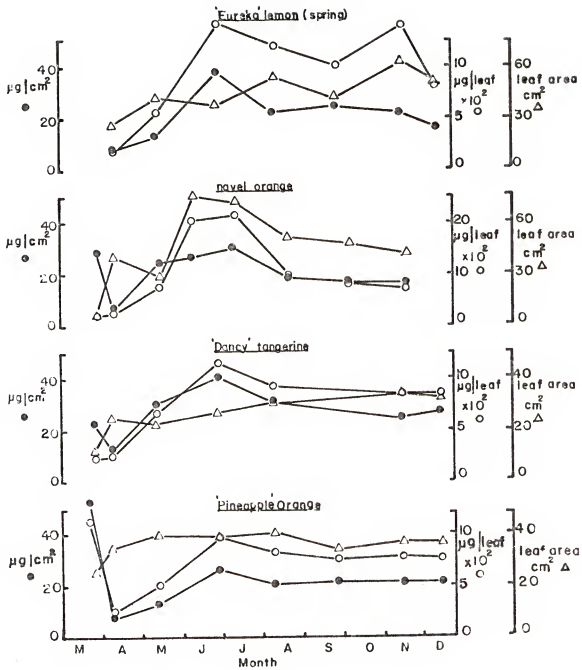
Surface waxes

Leaves.

(a) Citrus. Wax density refers only to the weight of wax per unit leaf or fruit surface area in all subsequent discussions. It does not allude to specific gravity or any other physical constant.

The wax density of each cultivar initially declined (Figure 65). This was not observed with the spring flush leaves of 'Eureka' lemon due to the lateness of the first sample. The same decline was observed, however, with summer flush 'Eureka' lemon leaves, the density value

Figure 65. Changes in the surface area, total wax per leaf and total wax per unit surface area of four citrus cultivars sampled between March 24 and December 15, 1977.


'Eureka' lemon (spring)
navel orange
'Dancy' tangerine
'Pineapple' Orange
μg/cm²
μg/leaf x10²
leaf area cm²
M A M J J A S O N D
Month

declining from 28.9 $\mu g/cm^2$ to 12.3 $\mu g/cm^2$ between late June and early August (Table A-5). The decline is due in each case to rapid leaf expansion, the rate of which exceeded the rate of wax accumulation. The expression "wax accumulation" is used to denote the net amount of wax on the surface balanced by synthesis, deposition and loss factors such as weathering, reentry to the epidermis [24, 25] and microorganism attack. Total wax per leaf declined on 'Eureka' lemon (summer flush) (Table A-5) and 'Pineapple' orange between the first and second samplings (Figure 65) and this reinforced the decline in density. Thus, the initial rate of wax accumulation was so low in these two cultivars as to produce a net loss in total wax per leaf. Wax per leaf remained relatively constant initially in navel orange and 'Dancy' tangerine. However, in all cultivars, the rates of leaf expansion exceeded the rates of wax accumulation and, hence, wax density was reduced. The rate of wax accumulation increased from early April until late June with a resultant increase in total wax and wax density. This coincided with the period of final leaf expansion or soon afterwards. Total wax and wax density declined subsequent to the June peak of wax production. This occurred until mid-August with wax levels remaining constant thereafter. Baker et al. [16] recorded peaks in citrus leaf wax production for four cultivars 3 to 4 weeks following full leaf expansion after which values declined slightly. None of the cultivars exhibited the initially higher density values followed by a rapid decline as described here. Leaf wax densities described here, 10 to 54 $\mu g/cm^2$, are similar to those reported by Baker et al. [16] for four citrus cultivars, 10 to 40 $\mu g/cm^2$. Other studies have reported leaf wax densities for 'Valencia' orange of 12 to 18 $\mu g/cm^2$ [108] and 30 to 40

$\mu g/cm^2$ [3]. The wide variation may be attributed to the time of sampling and the environmental conditions during leaf development [4]. Late spring and summer weather in Florida is characterized by high relative humidity, high temperatures and frequent intense showers of rain. This contrasts with the drier Mediterranean type climates in citrus growing regions of California and Greece. Thus, differences in leaf wax production reported from different areas may be largely influenced by climate.

(b) Blueberry. Blueberry leaf wax density initially declined and then rose to a peak of 138 $\mu g/cm^2$ in mid-May (Figure 66). Total wax increased steadily and rapidly from March to mid-May. Full leaf expansion occurred between late April and early May (Figure 66). Both wax density and total wax declined by 50% and 44%, respectively between mid-May and mid-August. It is considered that weathering may be the greatest single factor contributing to this loss.

Fruit.

(a) Citrus. Only the navel orange fruits of the three citrus cultivars showed a decline in wax similar to that described for leaves during the May-June period (Figure 67). This was due to the rate of wax accumulation remaining constant while the fruit underwent rapid expansion. The rate of wax accumulation increased sufficiently from mid-June to maintain a constant increase in wax density from 28 to 91 $\mu g/cm^2$ by November. Both 'Dancy' tangerine and 'Pineapple' orange fruits were accumulating wax at higher rates, sufficient to increase wax density during May-June. With both cultivars, however, the rate declined in August and continued fruit expansion caused a net decline

Figure 66. Changes in the surface area, total wax per leaf and total wax per unit surface area of 'Bluegem' blueberry leaves sampled between March 11 and October 6, 1977.

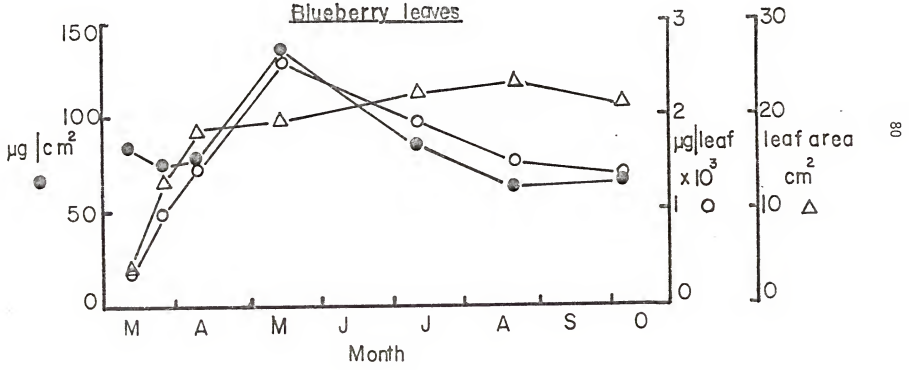
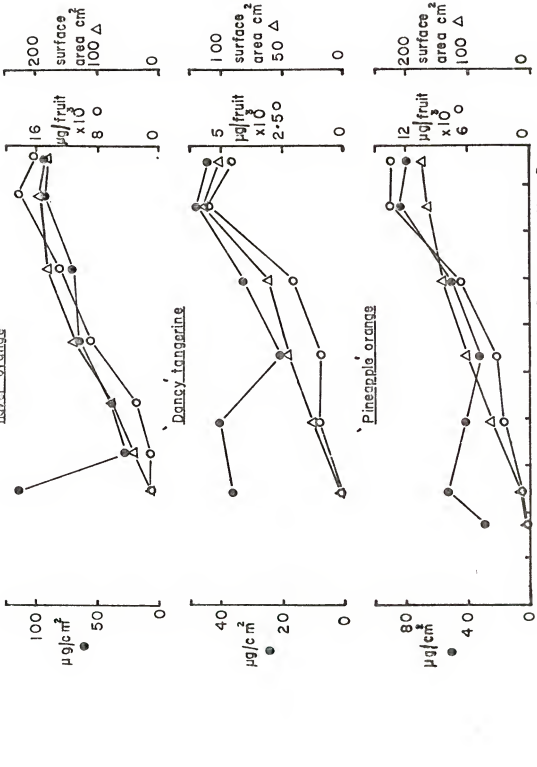

Blueberry leaves
150
100
50
0
µg / cm²
3
2
1
0
µg/leaf x 10³
30
20
10
0
leaf area cm²
M
A
M
J
J
A
S
O
Month

Figure 67. Changes in the surface area, total wax per fruit and total wax per unit surface area of three citrus cultivars sampled between March 24 and December 15, 1977.

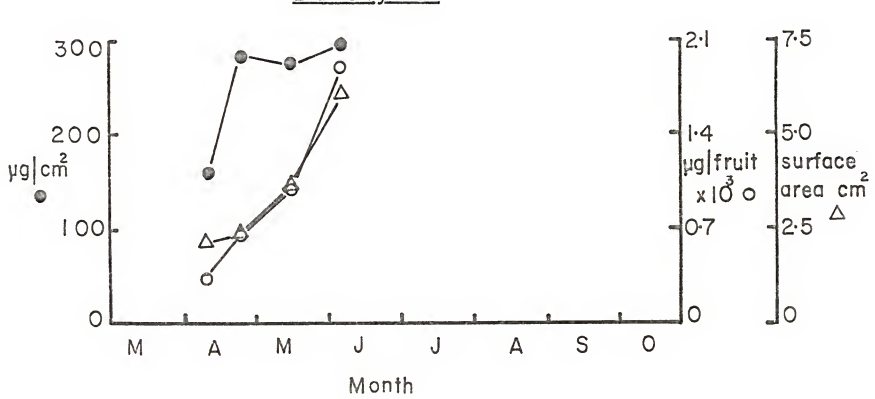

navel orange
Dancy tangerine
Pineapple orange
µg/cm²
µg/fruit x10³
surface area cm²
M A M J J A S O N D
Month

in wax density. From August to November wax accumulation rates and wax densities increased from 32 to 84 $\mu g/cm^2$ and from 21 to 48 $\mu g/cm^2$ for 'Pineapple' orange and 'Dancy' tangerine fruit, respectively. Baker et al. [16] described similar wax production curves for four different citrus fruit cultivars, i.e., two peaks in wax density 4 to 6 months apart. Wax production curves for navel orange fruits are similar to those described for 'Valencia' orange [2] and 'Shamouti' orange [157] fruit, both of which had greater than 70 $\mu g/cm^2$ at maturity.

Differences in wax densities among cultivars were greater in fruits than leaves. This is in agreement with the observations of Baker et al. [16]. Maximum values for mature 'Pineapple' and navel orange fruits, 80 to 90 $\mu g/cm^2$, were almost double those of 'Dancy' tangerine, 48 $\mu g/cm^2$. The latter value agrees with those recorded for 'Dancy' tangerine, 40 to 53 $\mu g/cm^2$, in 1972 in Florida [4]. Geographical location has been shown to affect fruit wax densities. Wax densities of 92 to 154 $\mu g/cm^2$ compared with 174 to 186 $\mu g/cm^2$ were reported for Florida and California grown 'Valencia' orange fruit, respectively [4]. Values for the same cultivar ranged from 50 $\mu g/cm^2$ in Greece [16] to 73 $\mu g/cm^2$ in Israel [157] and 160 $\mu g/cm^2$ in Australia [50]. Consideration should also be given to differences in techniques of individual researchers. Valid comparisons can only be made when the extraction and isolation methods are rigidly controlled.

(b) Blueberry. Blueberry fruit surface area increased rapidly between April and June (Figure 68). Wax accumulation was initially able to keep pace with this resulting in an increased wax density. By late April, however, wax density had reached a peak and was relatively

Figure 68. Changes in the surface area, total wax per fruit and total wax per unit surface area of 'Bluegem' blueberry fruits sampled between April 9 and June 5, 1977.


Blueberry fruit
300
200
100
0
µg/cm²
2·1
1·4
0·7
0
µg/fruit x10³ o
7·5
5·0
2·5
0
surface area cm² △
M
A
M
J
J
A
S
O
Month

constant thereafter. This occurred despite a continuing rapid rate of wax accumulation and may be accounted for in terms of continued rapid fruit expansion and some possible weathering of the wax rodlets. These were shown to be more delicate than other wax forms by their susceptibility to beam damage at 20kv in the SEM.

The difficulty in correlating quantitative (Figures 65-68) with qualitative data (Figures 1-64) can be appreciated when differences in sample size are considered. Several hundred leaves and fruits contributed to a single quantitative value while, at best, only portions of a few leaves and fruits could be examined by SEM at each of the sampling dates. While Figures 1-64 aimed to be as representative as possible of material observed, it was not possible to indicate all of the variability of each of the changes in wax structure. The observed cracking and lifting of the surface waxes in leaves occurred in general, therefore, subsequent to the peak in wax production, i.e., from August onward. The apparent loss of the wax plates was more prevalent in October and November. Thus, if weathering was indeed occurring, then the leaves must have been accumulating new wax through to November in order to maintain the relatively constant wax density values. The periods of maximum leaf wax production are seen prior to surface breakdown when densities appear to be greatest. These are shown in Figures 4, 13, 21, 27 and 34-36 for 'Pineapple', 'Dancy' tangerine, navel, 'Eureka' lemon and blueberry, respectively. In contrast, cracking, lifting and plate formation of fruit waxes occurred prior to maximum wax production. Fruits of 'Pineapple', 'Dancy' tangerine, navel and blueberry are represented in Figures 44, 50, 55 and 63, respectively, at their maximum wax densities in November and June (blueberry).

Cutin and embedded waxes

Removal of embedded waxes from isolated leaf and fruit cuticles resulted in similar percentage weight losses for all of the citrus cultivars except 'Dancy' tangerine fruit and blueberry (Table 2). Weight loss can be attributed to the removal of any remaining cellular debris and embedded waxes. The latter were retrieved as the hexane soluble fraction following refluxing. The constancy of weight loss, therefore, reflects both constant cellular debris (not calculated) and embedded wax densities values. All of the cuticles examined had embedded wax values within the range 33 to 40 $\mu g/cm^2$ with the exception of blueberry leaf cuticle, 22.5 $\mu g/cm^2$ embedded wax and navel orange fruit cuticle, 58.0 $\mu g/cm^2$ embedded wax. Leece [108] reported 43 to 48 $\mu g/cm^2$ for 'Valencia' leaves and Baker and Procopiou [15] recorded a range of 77 to 87 $\mu g/cm^2$ for four other citrus cultivars. The latter values are higher than those presented here but they do concur in showing that embedded waxes are usually greater in density than surface waxes. This is an observation contrary to previous studies [9, 76].

Cutin was taken to be that remaining following the removal of embedded waxes from isolated cuticles. This is not pure cutin as cuticles extracted this way still contain traces of cellulose and polysaccharides [74]. Impure leaf cutin values ranged from 206 to 322 $\mu g/cm^2$, values close to those recorded for 'Valencia' leaves, 370 to 387 $\mu g/cm^2$, [108] and four other citrus cultivars, 280 to 316 $\mu g/cm^2$, [15]. Fruit cutin values, 210 to 451 $\mu g/cm^2$, tended to be greater than those for leaves. This concurs with previous reports [78] but is contrary to the data of Baker and Procopiou [15] who found the reverse to

Table 2. Weight and wax content of isolated citrus leaf and fruit and blueberry leaf cuticles.

	Cuticle weight loss[z] (%)	Cutin[y] ($\mu g/cm^2$)	Embedded wax ($\mu g/cm^2$)	Surface wax ($\mu g/cm^2$)
Leaves				
'Pineapple' orange[x]	18.3 (3.5)[w]	310.4 (13.6)	33.7 (3.4)	20.6 (1.1)
'Dancy' tangerine	19.1 (3.4)	310.6 (35.1)	36.4 (3.0)	31.0 (0.1)
Navel orange*	15.8 (0.7)	322.4 (14.4)	33.9 (2.3)	19.4
'Eureka' lemon (spring)	19.0 (4.4)	281.1 (15.1)	33.7 (3.4)	22.1
'Bluegem' blueberry*	21.3 (1.7)	206.3 (12.1)	22.5 (1.2)	62.9
Fruit				
'Pineapple' orange	18.0 (1.4)	349.7 (39.8)	40.5 (7.7)	30.7
'Dancy' tangerine	30.5 (4.2)	210.2 (24.2)	33.2 (12.1)	21.3 (1.4)
Navel orange	21.5 (3.4)	457.5 (64.8)	58.0 (1.6)	66.2

[z]Percentage loss in weight of dewaxed cuticles following removal of embedded waxes by reflux.

[y]Cutin expressed as that left after removal of all surface and embedded waxes.

[x]All data were from samples collected August 10 unless otherwise denoted (*) in which samples were collected August 21.

[w](± S.E.).

be true. There was no correlation between values of cutin and embedded or surface waxes.

Chemical Data

Identification of lipid classes

The wax classes were identified on the basis of Rf values and co-chromatography with known standards (Table 3). β-Diketones from blueberry leaf and fruit wax showed strong absorption in the ultraviolet, λ max at 278 nm in chloroform [80] and a strong band between 1600 and 1650 cm^{-1} in infrared spectra (KBr) [80]. The Lieberman-Burchard test [79] produced mauve spots for triterpenyl acetate and citrus triterpenoids and a red-brown spot for blueberry triterpenoid. The blueberry triterpenoid had characteristic infrared bands at 3420 and 1690 cm^{-1} [108, 141]. Depsite the difference in spot color with Lieberman-Burchard reagent, the triterpenoids of blueberry and citrus both had Rf values of 0.65 when developed in chloroform:ethylacetate (1:1) v/v mixture. This was the same Rf value of ursolic acid which was tested concurrently.

Soft waxes

The proportion of soft waxes declined in all of the citrus fruit and leaf surface waxes and blueberry leaf wax from approximately 80% in March to 20 to 40% in November (Table 4). This is very similar to changes observed by Albrigo [2] for 'Valencia' orange peel wax. Thin layer chromatography showed soft waxes to contain all of the wax constituents. TLC of the hard wax fraction, however, indicated only primary alcohols, fatty acids and triterpenoids were present. Hard waxes

Table 3. Rf values of epicuticular wax constituents.

Constituent	Rf[z]
Paraffins	0.85
Esters	0.78
Ketones	0.73
β-Diketones	0.73
Aldehydes	0.65
Secondary alcohols	0.50
Triterpenyl acetate	0.35-0.4
Primary alcohols	0.15
Fatty acids	0.08
Acidic triterpenoid	0.01-0.02

[z]Spots developed on Silica gel G, 250 μ with benzene:acetic acid (99:1) v/v.

Table 4. Changes in percentage "soft" wax of citrus and blueberry leaf and fruit epicuticular waxes.

	Mar 11	Mar 24-26	Apr 9	Apr 22-24	May 12-15	June 8	June 28
Leaves							
'Pineapple' orange	---	81.7 (5.0)[z]	71.1 (4.9)		53.4 (7.9)		62.9 (3.5)
Navel orange	---	79.3 (0.8)	50.1		48.6 (4.4)	43.9	
'Dancy' tangerine	---	82.9 (6.8)	71.3		61.4 (12.5)		42.3 (2.6)
'Eureka' lemon	---	---	46.1		34.2 (4.6)		50.5
'Eureka' lemon summer flush	---	---	---	---	---	---	72.8 (2.3)
'Bluegem' blueberry	66.1	62.9	36.7	---	45.1		
Fruits							
'Pineapple' orange	---	---	---	58.9	73.3 (6.2)		60.2 (7.9)
Navel orange	---	---	---	---	80.5		
'Dancy' tangerine	---	---	---	---	67.7		58.6
'Bluegem' blueberry	---	---	---	41.9	43.0	55.3	

[z](± S.E.)

Table 4. Continued.

	July 10	Aug 10	Aug 21	Sep 27	Oct 6	Nov 15-20	Dec 15
Leaves							
'Pineapple' orange		28.2 (6.0)[z]		34.6 (3.5)		33.4 (4.6)	50.3 (4.1)
Navel orange	46.8		35.8		42.5	47.7 (1.1)	
'Dancy' tangerine		37.0 (2.9)		34.9		28.3 (3.1)	38.4
'Eureka' lemon		30.2		38.9		35.9 (0.8)	35.3 (0.0)
'Eureka' lemon summer flush		31.5		36.1		36.7	
'Bluegem' blueberry	35.4				25.1		
Fruits							
'Pineapple' orange		29.9		24.6 (4.8)		24.8 (3.6)	35.0 (2.1)
Navel orange	56.4		33.3		40.6 (1.4)	35.7 (0.1)	31.1 (2.4)
'Dancy' tangerine		21.6 (1.8)		25.2 (0.8)		14.6 (0.2)	28.5 (2.1)
'Bluegem' blueberry							

[z](± S.E.).

were defined as acidic materials, alcohols and ketones [163] and the observations here concur, with the exception of ketones. However, the distinction between hard and soft relates to solubility in petroleum ether [163]. Thus, Radler and Horn [140] found that hard waxes of grape wax were triterpenoids only. The reason for primary alcohols being present in both hard and soft waxes in these studies is not clear. It may relate to chain length or the presence of branched chains, both of which would influence solubility of the primary alcohol.

Chemical constituents of surface waxes

Changes in the wax constituents of *Citrus* leaf and fruit waxes are presented in Figures 69-77. Leaf and fruit wax constituents of blueberry wax are shown in Figure 78.

Paraffins.

(a) Citrus. Paraffins were a major constituent of citrus leaf waxes (Figure 69) and ranked second in overall importance to primary alcohols in all of the cultivars except 'Pineapple' orange. Paraffin content varied from 2 to 67% of total leaf wax (Tables A-1 to A-5) among cultivars and stages of maturity. Values were generally less than those reported for mature 'Valencia' leaves, 40%, [108] and four other citrus cultivars, 23 to 42%, [16]. The paraffins of immature and mature citrus fruit comprised 30 to 44% and 6 to 12 of total wax, respectively (Tables A-6 to A-8). This compares with 23 to 42% reported for mature citrus fruit [16] and 11% for mature 'Shamouti' orange peel wax [157].

Figure 69. Changes in the leaf and fruit wax paraffins, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars.

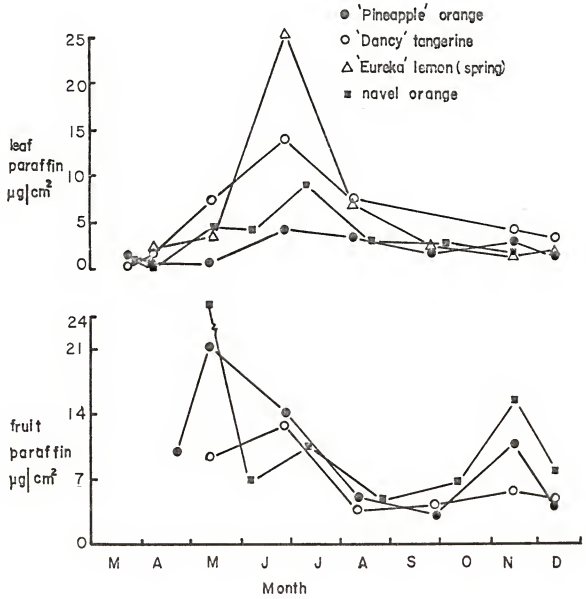

'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange
leaf paraffin µg/cm²
25
20
15
10
5
0
fruit paraffin µg/cm²
24
21
14
7
0
M A M J J A S O N D
Month

Figure 70. Changes in the leaf and fruit wax primary alcohols, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

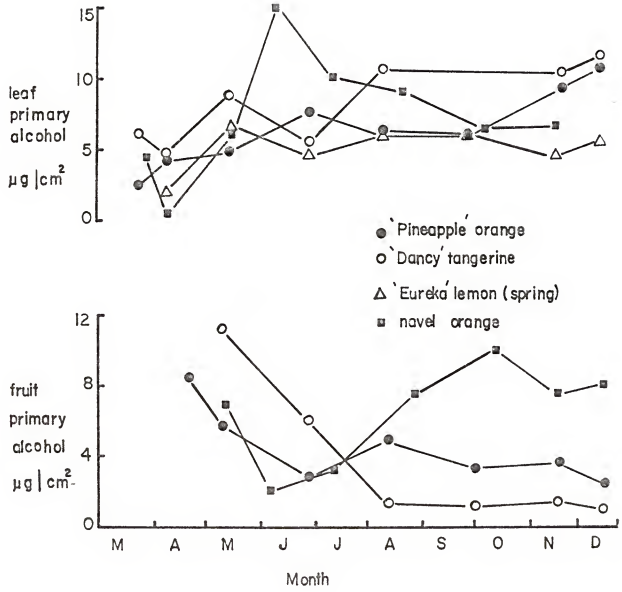
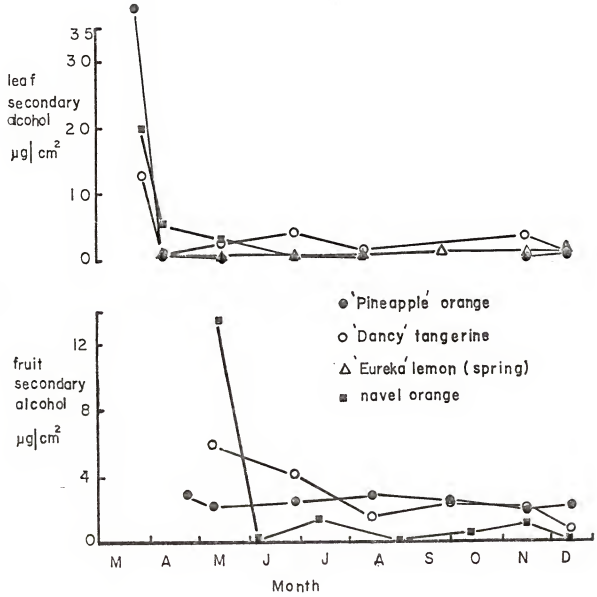

leaf primary alcohol µg/cm²
15
10
5
0
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange
fruit primary alcohol µg/cm²
12
8
4
0
M
A
M
J
J
A
S
O
N
D
Month

Figure 71. Changes in the leaf and fruit wax secondary alcohols, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.


leaf
secondary
alcohol
μg/cm²
35
30
20
10
0
fruit
secondary
alcohol
μg/cm²
12
8
4
0
M
A
M
J
J
A
S
O
N
D
Month
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange

99

Figure 72. Changes in the leaf and fruit wax aldehydes, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

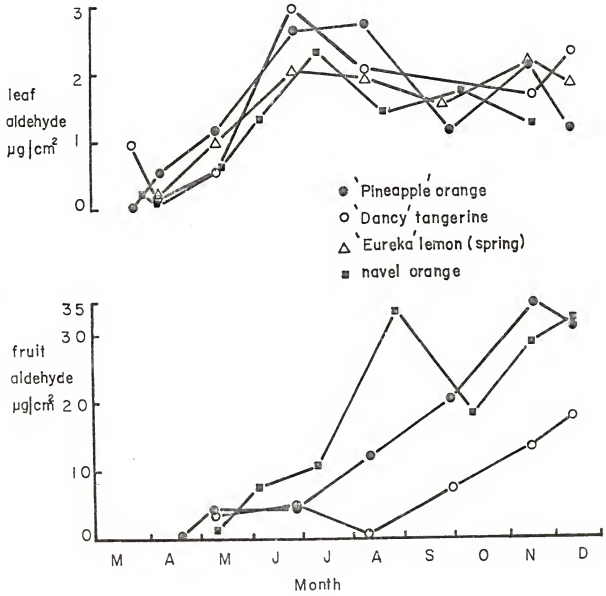

leaf
aldehyde
μg/cm²
3
2
1
0
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange
fruit
aldehyde
μg/cm²
35
30
20
10
0
M
A
M
J
J
A
S
O
N
D
Month

Figure 73. Changes in the leaf and fruit wax fatty acids, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

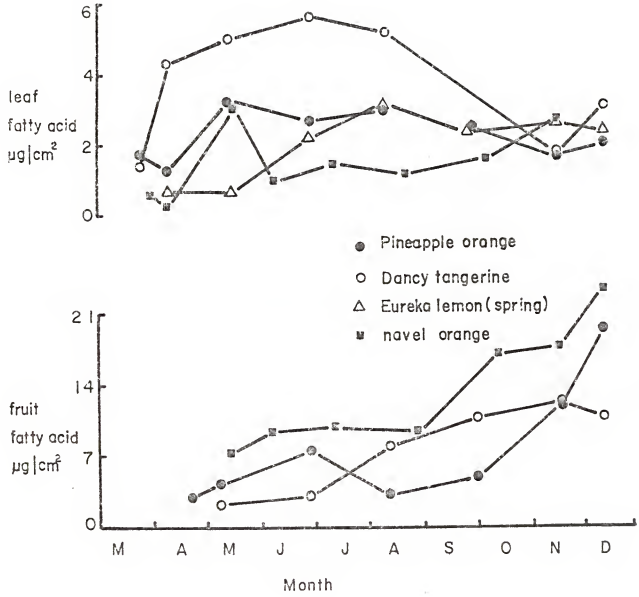

leaf
fatty acid
μg/cm²
6
4
2
0
fruit
fatty acid
μg/cm²
21
14
7
0
M
A
M
J
J
A
S
O
N
D
Month
Pineapple orange
Dancy tangerine
Eureka lemon (spring)
navel orange

Figure 74. Changes in the leaf and fruit wax ketones, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

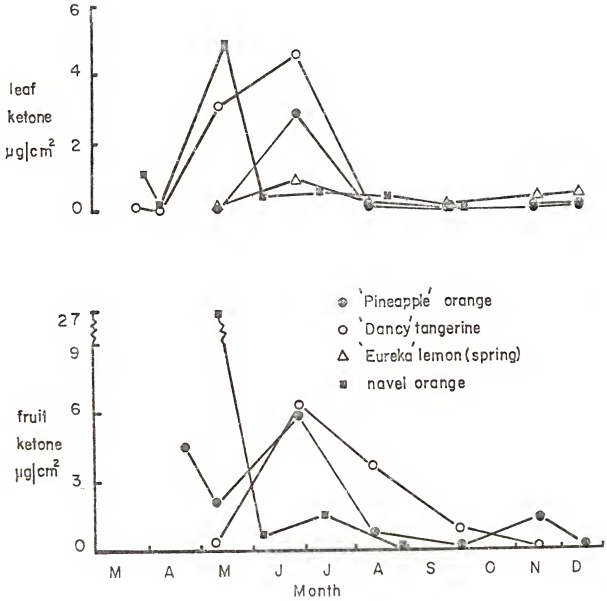

leaf
ketone
µg/cm²
6
4
2
0
fruit
ketone
µg/cm²
27
9
6
3
0
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange
M
A
M
J
J
A
S
O
N
D
Month

Figure 75. Changes in the leaf and fruit wax esters, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

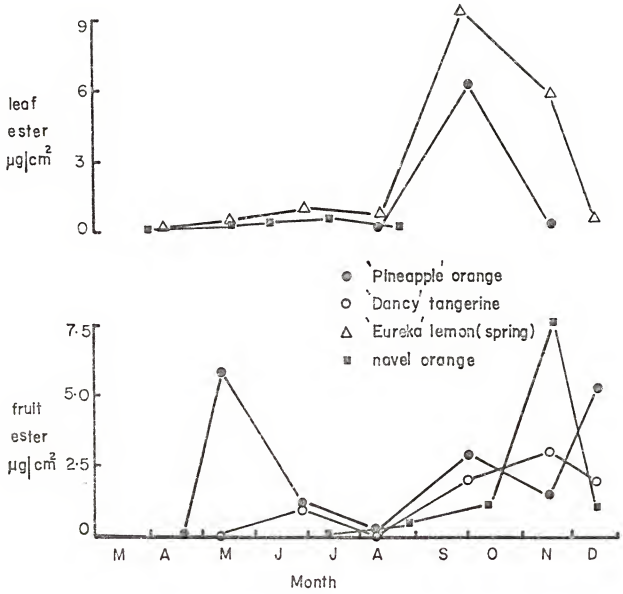

leaf
ester
µg/cm²
9
6
3
0
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange
fruit
ester
µg/cm²
7·5
5·0
2·5
0
M
A
M
J
J
A
S
O
N
D
Month

Figure 76: Changes jn the leaf and fruit wax triterpenyl acetates, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

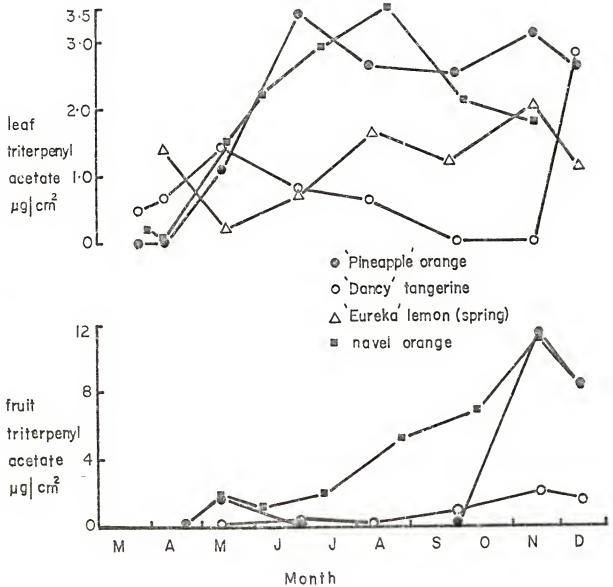

3·5
3·0
2·0
1·0
0
leaf
triterpenyl
acetate
µg/cm²
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
navel orange
12
8
4
0
fruit
triterpenyl
acetate
µg/cm²
M
A
M
J
J
A
S
O
N
D
Month

Figure 77. Changes in the leaf and fruit wax triterpenoids, expressed as $\mu g/cm^2$ surface area, of four citrus cultivars sampled between March 24 and December 15, 1977.

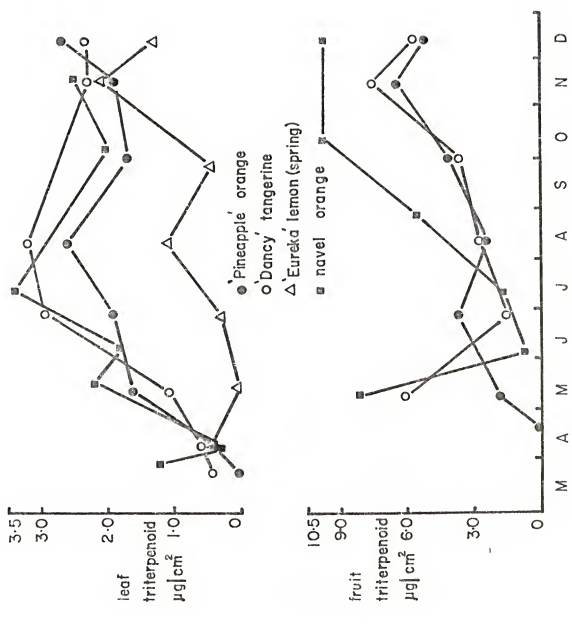
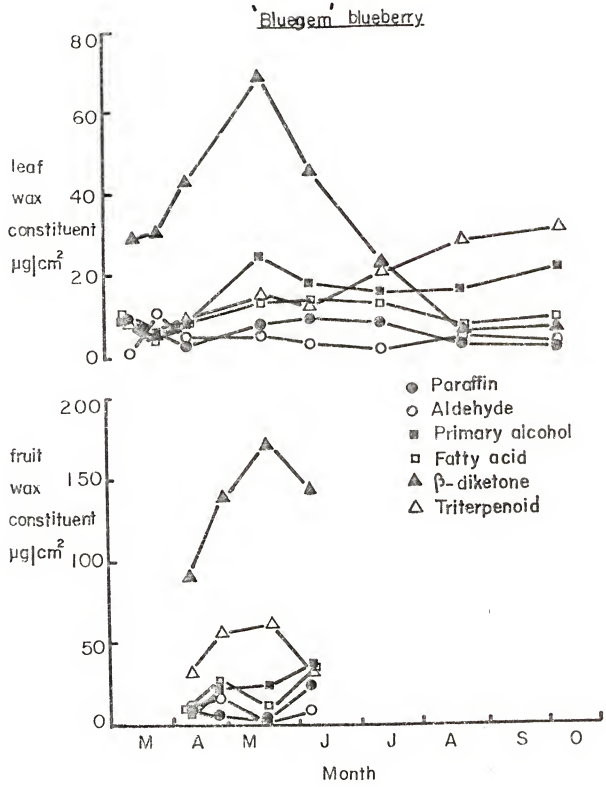

3·5
3·0
2·0
1·0
0
leaf triterpenoid µg/cm²
'Pineapple' orange
'Dancy' tangerine
'Eureka' lemon (spring)
105
90
60
30
0
fruit triterpenoid µg/cm²
navel orange
M A M J J A S O N D
Month

Figure 78. Changes in some leaf and fruit wax constituents, expressed as $\mu g/cm^2$ surface area, for 'Bluegem' blueberry sampled between March 11 and October 6, 1977.


'Bluegem' blueberry
leaf wax constituent µg/cm²
fruit wax constituent µg/cm²
0
20
40
60
80
50
100
150
200
M
A
M
J
J
A
S
O
Month
Paraffin
Aldehyde
Primary alcohol
Fatty acid
β-diketone
Triterpenoid

Changes in percentage composition do not reflect real changes due to variation of total wax with time. Real changes are best expressed as total paraffin (Tables A-1 to A-8) or paraffin density (expressed as $\mu g/cm^2$ surface area) (Figure 69). Leaf wax paraffin was low until early to mid-May (Figure 69). Density increased thereafter to a maximum in late June to early July. This peak coincided with that of total wax production described previously and was greatest for spring flush 'Eureka' lemon leaves and 'Dancy' tangerine leaves (Figure 69). During this period, paraffins were the greatest contributors to total wax in these two cultivars. Paraffin density declined in all cultivars following the June peak. This decline was greatest for 'Dancy' tangerine and 'Eureka' lemon leaves. 'Eureka' lemon leaf wax paraffins declined from 25 to 2.5 $\mu g/cm^2$, a tenfold reduction which was greater than that for total wax. The deficit was made up by increases in other wax constituents (Figures 70, 73, 76, 77).

Fruit wax paraffins (Figure 69) also had peak densities in early summer. These coincided with the peaks in leaf wax paraffins for 'Dancy' tangerine and navel orange. The paraffin peak for 'Pineapple' orange occurred earlier in May. Fruit wax paraffin density declined between June and August in all cultivars (Figure 69), as did total paraffin per fruit (Tables A-6 to A-8). The decline in density may be attributed to the rapid increase in fruit surface area. However, in navel orange, total wax density and total wax increased during the period of paraffin decline. In the other two cultivars total wax remained constant while total paraffin declined. Thus, there appears to be a net loss of paraffin from the fruit waxes. Whether this was due to weathering or reabsorption [24, 25] is not clear. Paraffin densities and total

paraffin (Tables A-6 to A-8) again increased from September (Figure 69) to give a secondary peak in November, followed by another decline.

(b) Blueberry. Paraffins were a minor constituent of blueberry leaf and fruit waxes, 1 to 10% (Tables A-9, A-10). In both cases there was a small peak in total paraffin (Tables A-9, A-10) and paraffin density (Figure 78) in June. Paraffin content of mature 'Howes' cranberry (*Vaccinium macrocarpon*) fruit, another *Vaccinium* species was 11% [35], as compared to 7% for blueberry.

Primary alcohols.

(a) Citrus. Primary alcohols were the dominant leaf wax constituent throughout the sampling period for 'Pineapple' and navel orange (Tables A-1, A-2). Primary alcohols were the dominant constituent in wax from 'Eureka' lemon and 'Dancy' tangerine leaves except for the period between June and July (Tables A-2, A-4) when paraffins were predominant. Primary alcohols ranged from 6 to 55% of the leaf waxes and 3 to 29% of fruit waxes (Tables A-1 to A-8). These values correspond closely with those reported for other citrus cultivars [16, 108] with the exception of 'Shamouti' orange peel wax in which alcohols and ketones combined amounted to less than 2.3% of total wax [157].

Rates of primary alcohol accumulation were low during the period of initial leaf expansion and densities declined in 'Dancy' tangerine and navel orange leaves (Figure 70). Between April and June, primary alcohol density of navel orange leaf wax increased from 1 to 15 $\mu g/cm^2$ and steadily declined thereafter until October. This strong peak in June coupled with a July paraffin peak was largely responsible for the

flattened June to July peak of total wax density described previously. The primary alcohol density of 'Pineapple' orange leaf wax peaked in late June (Figure 70), reinforcing the peak in paraffin density at this time. They jointly contributed to the June peak in total wax density. Primary alcohol density almost doubled in this cultivar between September and December (Figure 70). Density of leaf primary alcohol declined during June in 'Dancy' tangerine and 'Eureka' lemon (Figure 70). These values increased in August, were constant to November and increased slightly in December. The June decline in primary alcohol density for these two cultivars would have compensated for their large June peaks in paraffin density with respect to total wax.

Primary alcohol densities initially declined in all citrus fruit cultivars (Figure 70), a consequence of rapid fruit expansion. Total primary alcohol, however, peaked in June for 'Dancy' tangerine fruit and was relatively constant thereafter (Table A-7). This suggests a rate of accumulation equal to the rate of fruit growth. The total primary alcohol of 'Pineapple' orange fruit wax increased slowly until August (Table A-6) and was constant thereafter, being similar to 'Dancy' tangerine. The rate of primary alcohol accumulation was greatest in navel orange. Total primary alcohol per fruit (Table A-8) and density (Figure 70) increased until October, after which a slight decline was observed.

(b) Blueberry. The primary alcohol density of blueberry leaf wax increased until mid-May (Figure 78), the period at which total wax density was greatest. It remained relatively constant thereafter despite a decline in total wax density. Primary alcohols were a minor

constituent of blueberry fruit wax (Figure 78). Accumulation rate was sufficient to maintain increasing density, despite the rapid rate of fruit expansion.

Secondary alcohols.

(a) Citrus. Secondary alcohols were the most dominant wax constituent in immature citrus leaves, comprising up to 86% of total leaf wax (Table A-1). Previous studies have not reported secondary alcohols to be constituents of citrus leaf waxes [16, 108] as they were concerned only with mature leaves. This predominance of secondary alcohol was quickly lost by April, however, and subsequent samplings showed little or no secondary alcohol in citrus leaf waxes (Figure 71). Secondary alcohols were a minor component of citrus fruit waxes (Tables A-6 to A-8) although initially high in navel orange fruit wax (Figure 71). The fate of these secondary alcohols is not clear. Either they were rapidly weathered, reabsorbed or were a consequence of the extraction procedure. It is possible that initial extractions of immature leaves were removing some intracellular lipids, principally secondary alcohols. Steps were taken to avoid this when navel orange fruits were first extracted. Waxes extracted by five successive, 10 sec immersions in chloroform were kept separate. Secondary alcohols were abundant in all of the extracts, including the first, suggesting they were part of the surface wax. Fruit wax secondary alcohols remained at low but constant levels between June and December (Figure 71). Therefore, the rate of accumulation apparently equalled the rate of fruit expansion.

(b) Blueberry. Secondary alcohols of blueberry leaf wax were second in importance to β-diketones in March (Table A-9). There were no secondary alcohols in the leaf wax by April, however, a situation paralleling that of some citrus leaves. The secondary alcohol content of blueberry fruit wax was minor, ranging from 0.7 to 2.6% of total wax (Table A-10).

Aldehydes.

(a) Citrus. Aldehydes were a minor fraction of citrus leaf waxes (Figure 72) being for the most part less than 10% of total wax (Tables A-1 to A-5). They were not observed in 'Valencia' leaf wax [108] and were less than 1% of total leaf wax in the four cultivars studied by Baker et al. [16]. Changes observed in leaf wax aldehyde were similar for all the cultivars studied. There was a constant rise in density from April until June to July and a gradual decline thereafter (Figure 72). Thus, each cultivar had equivalent rates of aldehyde accumulation and probably synthesis.

Aldehydes were the major fruit wax component in 'Pineapple' and navel oranges from July to December; and in 'Dancy' tangerine from October to December (Tables A-6 to A-8). The density of aldehyde in the first two cultivars increased continuously from June, while the increase commenced in August in the latter (Figure 72). Aldehydes were the dominant fractions in mature fruits, comprising 20 to 50% of total wax (Tables A-6 to A-8). This compares with a range of 28 to 44% for four other citrus cultivars [16].

(b) Blueberry. Aldehydes were a minor fraction of both blueberry leaf and fruit waxes (Figure 78).

Fatty acids.

(a) Citrus. Fatty acids were minor in importance in citrus leaf waxes and changed only slightly during the period of sampling (Figure 73). 'Dancy' tangerine leaf wax fatty acid values were double those of the other cultivars tested, and a June maximum was followed by a decline through to November. Fatty acids ranged from 4 to 17% of total wax in all the cultivars tested (Tables A-1 to A-5). This compares with 2% for 'Valencia' leaf wax [108] and 2 to 20% for four other citrus cultivars [16].

Fatty acids were an important fraction of citrus fruit waxes, comprising 24 to 25% of total wax at fruit maturity (Tables A-6 to A-8). This compares with 7% for 'Shamouti' orange [157] and 8 to 20% for four other citrus cultivars [16]. Fatty acid content increased with fruit maturity (Figure 73). They were the second most abundant wax class in navel orange and 'Dancy' tangerine from July and from September in 'Pineapple' orange (Tables A-6 to A-8).

(b) Blueberry. Fatty acids increased slowly in blueberry leaf wax to a peak in June and declined only slightly thereafter. Fruit wax fatty acids peaked in April and declined temporarily in May probably due to rapid fruit growth and peaked again in June (Figure 78).

Ketones.

(a) Citrus. The density of leaf wax ketones in navel orange peaked in May at 5 $\mu g/cm^2$ (Figure 74) and thereafter declined to trace proportions. The other three cultivars had smaller peaks but these all occurred in June coincident with the peak in total wax. Navel orange fruit wax was rich in ketone, 21%, only at the first sampling but in trace amounts otherwise (Figure 74). Both 'Dancy' tangerine and 'Pineapple' orange fruit wax ketones peaked in June in similar fashion to the leaf ketones (Figure 74). Ketones have not been reported for citrus leaf or fruit waxes [16, 108] although ketones and alcohols together comprised 2.3% of 'Shamouti' orange peel wax [157].

(b) Blueberry. Ketones were not detected in blueberry fruit waxes and only in trace quantities on two occasions in leaf waxes (Tables A-9, A-10).

Esters.

(a) Citrus. Esters were mostly minor constituents of the citrus leaf waxes (Figure 75). Ester density increased between September and November in 'Eureka' lemon leaves and 'Pineapple' orange leaf and fruit waxes and declined again to trace levels. This rapid appearance and disappearance of ester was rechecked several times but it remained consistent. Leece [108] reported that the combined esters and alcohols of 'Valencia' leaf wax were 50% of total wax. Schulman and Monselise [157] found that esters comprised 66% of total 'Shamouti' orange peel was, whereas no esters were found in four other citrus cultivars [16].

(b) Blueberry. Esters were not detected in blueberry leaf wax (Table A-9). They were detected in the fruit wax being 5% of total wax, but only at the final sampling period (Table A-10).

Triterpenyl acetates.

(a) Citrus. Triterpenyl acetates generally were minor constituents of both citrus leaf and fruit waxes (Figure 76). However, they did represent a more significant proportion of the mature fruit and leaf waxes of navel, 9 and 18%, respectively (Tables A-3, A-8) and 'Pineapple' orange, 14 and 15%, respectively (Tables A-1, A-6). Leaf and fruit triterpenyl acetates ranged between 3 to 3.5 $\mu g/cm^2$ and 8 to 12 $\mu g/cm^2$, respectively, for these two cultivars (Figure 76). Values for the other cultivars were less than 2 $\mu g/cm^2$. Triterpenyl acetate accumulation in navel orange fruit wax increased from May to November at a rate similar to that of the total wax (Figure 76), but 'Pineapple' orange fruit triterpenyl acetate appeared only after September.

(b) Blueberry. Triterpenyl acetate accounted for less than 6% of total wax in blueberry leaves and fruit and were usually less than 2% (Tables A-9, A-10). In cranberry fruit wax they accounted for 6.7% of total wax [35].

Acidic triterpenoids.

(a) Citrus. Acidic triterpenoids were minor fractions in citrus leaf waxes being 1 to 15% of total wax (Tables A-1 to A-5). All cultivars, with the exception of 'Eureka' lemon, had similar quantities (Figure 77). Changes in their densities were almost superimposable over

those described previously for leaf wax aldehydes (Figure 72). By November all four cultivars had similar quantities of acidic triterpenoid in the leaf waxes, 1.9 to 2.5 $\mu g/cm^2$. Triterpenoids were not detected in citrus leaf or fruit waxes by Baker et al. [16], while Leece [108] found them to comprise 2% of 'Valencia' orange leaf wax.

The time-course change in acidic triterpenoids was similar to that of total wax in citrus fruit (Figure 77). Values were low in June and then increased to a maximum in November.

(b) Blueberry. The blueberry acidic triterpenoid fraction similar to that in citrus had Rf values of 0.01, 0.01 and 0.65 when developed in benzene:acetic acid (99:1), benzene:chloroform (7:3) and chloroform:ethyl acetate (1:1), respectively, corresponding with ursolic acid.

Blueberry leaf wax acidic triterpenoids increased in concentration throughout the entire sampling period (Figure 78) and became the dominant leaf wax fraction after July. Total acidic triterpenoid per leaf increased until late August, then remained constant (Table A-9). Total leaf wax, however, declined during this period.

Acidic triterpenoid was the second most important blueberry fruit wax fraction, increasing until May after which it declined (Figure 78). The decline was due to a reduction in the rate of accumulation as total per fruit remained constant during the same period (Table A-10). The triterpenoids and fatty acids of cranberry fruit wax, together comprised 40% of total wax [35]. These triterpenoids were a mixture of α-amyrin, β-amyrin, ursolic acid and oleanolic acid.

β-Diketones. These were exclusive to blueberry leaf and fruit waxes and were the major fraction in both cases (Figure 78).

Density increased on the leaf until it reached a peak in mid-May, coincidental with the peak in total wax. β-Diketone declined rapidly from 68 to 8 $\mu g/cm^2$, from mid-May to mid-August, while total leaf wax declined from 135 to 60 $\mu g/cm^2$ during the same period. Thus, most of the reduction in leaf wax density was attributed to loss of β-diketone. β-Diketone density in fruit wax increased until mid-May and then declined between May and June (Figure 78). This decline was due to fruit expansion as total β-diketone per fruit increased throughout the sampling period (Table A-10), as opposed to actual loss on leaves.

Albrigo [2] suggested that "hard" waxes might be inelastic, brittle waxes which would crack and form plates on citrus fruit surfaces. Conversely, "soft" waxes might be more pliable and less likely to crack or separate under the stress of fruit enlargement. The increase in hard waxes observed by Albrigo [2] was also observed in these studies for all of the citrus leaf and fruit waxes in addition to blueberry leaf wax. Albrigo [2] stated that breaking up of the previously continuous surface wax layer apparently depends upon hardening of this layer. This was said to be more likely a consequence of long exposure to the atmosphere rather than extrusion of hard wax which Albrigo stated may be more difficult to extrude [2]. More recent information makes it unlikely that "hardness" and ease of extrusion are equated. Hardness relates to specific chemical groups, alcohols, acidic and triterpenoid fractions, which as a group have higher melting points than "soft" waxes [163]. If we assume that waxes reach the surface in solution then all wax constituents should reach the surface with equal ease.

Albrigo [4] exposed some extracted wax to air for 30 days and the percent soft wax declined from 57 to 31%, while no change was observed

in the extracted control wax held at 0° C in N_2. Similarly, Purdy and Truter [138] extracted waxes from *Brassica* leaves and found that auto-oxidation occurred within 13 days, changing the chemical composition of the wax. However, surface wax on dried leaves held under the same conditions showed no change. Thus, the change in hardness *in vitro*, reported by Albrigo [4], may have been possible only after extraction, and opens to question whether auto-oxidation can cause changes of hardness *in vivo*. The correlation between ultrastructure, i.e., surface cracking to form plates, and wax hardness [2] may not necessarily be valid. Blueberry leaf wax showed a similar increase in hard wax as the citrus cultivars but no similarity existed with respect to ultrastructure. Albrigo [2] also equated cracking of the wax layer and subsequent plate formation with stresses induced primarily by fruit expansion. These stresses were present throughout the period of growth and yet cracking occurred only towards the latter stages. This may be explained if "hard" waxes are indeed more brittle as suggested by Albrigo [2]. Similarly, cracking and plate formation in the leaf waxes was observed subsequent to full leaf expansion when growth stresses would have been minimal or absent. However, diurnal changes in leaf thickness may be enough to cause cracking of brittle waxes. Shrinkage during air drying of the samples may be responsible also. This requires more research.

A consistent correlation does not exist between any specific wax chemical constituent and hard wax, hence, it is suggested that the degree of hardness relates to chemical groups and molecular structure. This is supported by the data in which these wax classes originally defined as hard, were identified by TLC in both the petroleum ether

soluble and insoluble extracts. Thus, solubility, and possibly plasticity, were based upon other factors besides wax class and these may include chain length, saturation and branching. Hydrocarbons of citrus leaf waxes increased from lower to higher carbon numbered alkanes upon fruit maturation [126]. Alkane chain length increased in citrus peel waxes [129] and juice sac waxes [130] as well as the relative percentages of monoene in total hydrocarbons [124]. Other species to show increasing epicuticular alkane chain length with maturity were *Hedera helix* [58], *Coffee arabica* [166] and *Solandra grandiflora* [72]. Haas [58] reported that primary alcohols in the surface wax of *H. helix* leaves increased in chain length with maturity and Baker and Holloway [12] demonstrated the presence of a substantial proportion of branched chain constituents in the alkyl ester and primary alcohol components of brussel sprout (*Brassica oleracea*) wax. Branched hydrocarbons are usually minor alkane components [128], but in some species, e.g., tobacco, they may be to 50% [184]. Citrus fruits are unique in having both iso- and anteiso-branched hydrocarbons [128]. No studies have been done to determine chain length, branching and desaturation changes in all of the major surface wax constituents with maturity. These types of data are required to better interpret the changes in wax ultrastrucutre and hardness.

If surface waxes are resistant to auto-oxidation *in situ* but not when extracted, the possibility exists then of bonding between the wax classes that renders them more inert. Extraction may destroy these bonds and auto-oxidation will proceed. The nature of these bonds and their strength may depend upon the spatial arrangement of chemical groups on the plant surface. This in turn may depend upon the sequence

of deposition of the various wax constituents. Jeffree et al. [89] clearly demonstrated that, for some plant waxes, their ultrastructure could only be reproduced by the wick-feed technique if the chemical groups were deposited sequentially on the surface. Thus, extracted waxes would not be expected to reform the same chemical bonds upon drying and their reactive groups may be more exposed. This may explain their susceptibility to auto-oxidation.

Amorphous waxes may be a heterogeneous mixture as suggested in the SEM figures previously described. Silva Fernandes et al. [163] reported that progressive extraction of apple fruit cuticles with chloroform indicated that paraffins and esters tended to be located in the surface layer. Possingham et al. [137] exposed grape berries to petroleum ether vapors and disorganized the platelet structure and removed most of the hydrocarbon, aldehyde and alcohol fraction. Similarly, Albrigo [1] exposed 'Hamlin' and 'Valencia' fruit surfaces to petroleum ether vapor and produced an etched surface on examination with SEM. Thus, it is postulated that the waxy surface which uplifted, cracked and formed plates may be more dominant in one chemical group, probably paraffins, then the underlying and occasionally surrounding areas. Between June and December, total leaf wax declined by varying amounts in three of four citrus cultivars. In all there was a net loss of paraffin and both paraffin and primary alcohol showed large losses in navel orange leaf wax. Other wax components remained constant or increased slightly. This indicates continuing wax synthesis with primary alcohols being the most common group, except in navel orange. If the paraffins are lost due to weathering, then the wax plates which are most likely to be removed from the surface are possibly rich in paraffins. Smooth wax flow

described on the leaf "ridges" may be newly extruded primary alcohols. Chang and Grunwald [29] observed that alkanes and diols of tobacco leaf wax were highest in young leaves and declined with age although total leaf wax remained constant. This preferential loss of wax components has not been explained and may be due to other factors discussed below in addition to or in place of weathering.

Leaf wax accumulation was not significant following full leaf expansion and the subsequent loss of wax constituents, although slight, was detectable. Fruit wax accumulation continued at a greater rate through November. Any loss of wax constituents by weathering could not be detected due to the greater rate of replacement. This may explain the greater loss in leaf paraffins after June than was detected in fruit. Cracking and plate formation was observed to occur as early as June to July in both fruit and leaf surfaces and may be considered support for the hypothesis that these surface plates are rich in paraffins. The relative contribution of individual wax constituents to amorphous wax ultrastructure has not been investigated. The influence of aldehydes and fatty acids in fruit waxes versus primary alcohols in leaf waxes requires further study. This also applies to the changes that occur in wax composition with time. Changes in the relative proportions of wax constituents with age were also reported for *Triticum* species [170]. Esters and β-diketones increased with age up to 66 days on leaf blades while alcohols declined. Alkane production continued throughout the life of the leaf in *Solandra grandiflora* [72], while wax composition changed during leaf development due to aldehyde formation in *Hedera helix* [58]. The surface wax of 'Cox's Orange Pippin' apples in storage

remained constant in amount and composition, whereas that of 'Bramley' fruits increased, specifically with respect to esters [11].

The data demonstrated that leaf wax synthesis continued in all samples after full leaf expansion in May to June. This was particularly evident on near senescent blueberry leaves upon which newly extruded wax rodlets were observed (Figure 37). Continued total wax synthesis was at a lower rate after leaf expansion although the rate varied among the different wax components. This is contrary to the concept of Schieferstein and Loomis [148, 149] who proposed that wax extrusion stopped when the leaf was mature, due to final hardening of the primary cuticle. Davis [38] also implied that wax extrusion ceased beyond a critical stage of growth. Giese [52] postulated that the amount of wax on the cuticle determines the rates of synthesis and extrusion. Thus, when a leaf has deposited surface wax to the level of its genetic potential under existing environmental conditions, synthesis and extrusion slow down or cease. This may also apply to the citrus cultivars studied. Replacement of lost wax, however, was too slow in the blueberry leaf to prevent a continued decline of total wax. The ability to replace lost wax must decline as leaves age if Giese's hypothesis is true. Loss of wax on blueberry leaves was mainly due to loss of the β-diketone component. This was compensated for by the production of new β-diketone in smaller amounts and triterpenoid in larger amounts. Hallam [66] removed surface wax from leaves of three *Eucalyptus* species and regeneration was complete in each case within 24 hours. This supports Giese's hypothesis of feedback control. However, it is not clear whether the stimulus for regeneration was a result of reduced wax levels or the mechanical action of wax removal.

Maximum rates of synthesis for leaf wax paralleled maximum rate in leaf growth. Following full leaf expansion an apparent lag period of high metabolic activity resulted in the June peaks of wax production. Both leaf and fruit wax paraffin and ketone synthesis increased and these wax groups contributed the most to the June peak. This and continued differentials in accumulation rates failed to suggest any product-precursor relationships. Fruit continued expanding to maturity and their sustained metabolic activity was reflected in increasing wax levels through to November. Continued synthesis in storage has been reported for citrus [157] and apple fruit [11] although at lower rates. Decline in synthesis after November may have been the result of a combination of maturity and environmental factors.

Changes in total wax, wax density and rates of synthesis for constituents have been described for leaves and fruits studied. The fate of those constituents which disappeared from the surface is not clear. The possibility of preferential weathering has been discussed but the possibility of reabsorption deserves consideration. Cassagne and Lessire used pulse chase to show reentry of fatty acids [25] and alcohols (personal communication) to epidermal cells where the former were decarboxylated to alkanes and redeposited. They indicate that a movement of wax components in and out of epidermal cells is likely and suggest that a steady state is established, not only between the wax and the internal lipids, but also among the different components of the wax on the surface. They suggest a new steady state tends to appear when environmental conditions change, adjusting the movement of wax substances, leading to a modification of the wax composition. Thus, loss of paraffin and appearance and loss of esters and ketones from citrus

waxes in addition to loss of β-diketones from blueberry leaves may be interpreted by a steady state concept modified by considerations of preferential weathering and possibly small amounts of auto-oxidation. Aldehydes are highly reactive and oxidize readily to acids (Biggs, personal communication). Both aldehydes and fatty acids accumulated at equivalent rates in citrus fruit waxes. Thus, a steady state concept could be envisaged as existing between these two dominant groups.

Chemistry of the intracuticular waxes

Embedded waxes were isolated from citrus leaves and fruits and from blueberry leaves. TLC indicated in all cases that they were predominantly fatty acids with minor proportions of paraffins and primary alcohols. These same wax classes were previously reported in leaf and fruit cuticles of several citrus cultivars [15] and the cuticles of spinach leaves [76]. Free fatty acids comprised 78 to 97% and 68 to 72% of total citrus leaf and fruit intracuticular waxes, respectively [15]. These were predominantly hexadecanoic acid with significant amounts of octadecanoic acid [15]. Surface waxes by contrast had fatty acids with mostly C_{24} to C_{34} chain lengths. C_{16} and C_{18} fatty acids were also predominant in the intracuticular waxes of strawberry [9], *Eucalyptus* [9] and spinach [76].

The role of the intracuticular fatty acids is not yet certain although there are several hypotheses. Holloway [76] reported that esterified monobasic acids in spinach cutin were similar in chain length to the free acids. Hexadecanoic acid was prominent in both, suggesting esterification of some of the intracuticular wax to form the cutin matrix. However, no free acids corresponding to the esterified

dibasic acid of the cutin occurred [76] indicating no definite chemical relationship between the intracuticular wax and cutin. Kolattukudy [105] showed that hexadecanoic acid is incorporated as the preferred substrate into the hydroxylated monomers of the cutin matrix. It is also the end product of *de novo* fatty acid synthesis and is the substrate for the formation of hydrocarbons [98], free long chain fatty acids, aldehydes, alcohols and esters [179]. Baker and Procopiou [15] suggest that localization of hexadecanoic acid near the boundary with the pectic layer may imply its involvement in the *in vivo* polymerization of cutin [104]. However, the location of intracuticular lipids has not been clearly established. Zones of negative birefringence were observed over anticlinal walls in the cutin matrix of pear cuticles [133]. It was suggested that this was embedded wax. Similarly, Albrigo (unpublished data) demonstrated islands of electron-dense material in the cuticle above the anticlinal walls of citrus leaves. Sargent [145] demonstrated a similar zone that stained positively with Alloxan/Schiff indicating protein. It was suggested that these were regions of active oxidation and polymerization of the cutin precursors, namely, C_{16} and C_{16} fatty acids. Cuticle lamellae observed in *Libertia* species were also proposed to be free fatty acids which are converted *in situ* to epicuticular waxes [146]. This seems to be most unlikely. Enzymes required for elongation occur in the cytosol [101] and cofactors NADPH and Malonyl-ACP have not been detected in cuticles. It is also unlikely that NADPH and $NADP^+$ would cycle between the cuticle surface and the cytosol of epidermal cells.

It is concluded, therefore, that hexadecanoic and octadecanoic acids may be involved in cutin polymerization but not in *in situ*

epicuticular wax synthesis. To date, no hypothesis has been proposed to explain the relative absence of C_{16} and C_{18} free fatty acids in surface waxes and why the surface wax extrusion mechanims precludes these from reaching the surface along with the other wax constituents.

Wax Extrusion and Manipulation

The wick-feed technique of Jeffree [88], but with excised citrus cuticles, proved to be successful in reproducing blueberry wax ultrastructure identical to that which occurs naturally. Citrus wax was reproduced equally well but with some reservations that will be discussed.

Wax Load

The optimal wax load (concentration) for blueberry wax was 0.55 mg/1.5 ml solvent. Loads which were too high caused the wax to form globular masses and clumps with poor rodlet differentiation (Figure 79). The arrangement and structure of the rodlets at 0.55 mg/1.5 ml solvent (Figure 80) was indistinguishable from natural blueberry leaf surfaces described previously. Rodlet density and size also progressively decreased when progressively lesser wax loads were applied (Figures 81, 82). The optimum load for citrus waxes was approximately 0.6 mg/1.5 ml solvent and this was used in all subsequent studies. These loads are similar to those determined by Jeffree et al. [89] for a range of other plant waxes.

Figures 79-82. Blueberry leaf wax deposited on isolated citrus leaf cuticles using a modified wick-feed technique after Jeffree [88]--effect of wax concentration.

Fig. 79. 1.1 mg wax/1.5 ml chloroform.

Fig. 80. 0.55 mg wax/1.5 ml chloroform.

Fig. 81. 0.28 mg wax/1.5 ml chloroform.

Fig. 82. 0.09 mg wax/1.5 ml chloroform.

79
3·1μm
80
1·8μm
81
2·8μm
82
2·1μm

Cuticle Type

Due to the difficulty of isolating intact blueberry cuticles, citrus cuticles were used throughout. Cuticles which were totally dewaxed were used unless otherwise specified. No difference in wax appearance was detected after extrusion through cuticles which were totally dewaxed or only surface dewaxed. Blueberry wax deposits that were extruded through adaxial cuticles are depicted in Figures 83 and 84. Lack of uniformity of deposition may be due to the lack of complete contact between cuticle and wick. Extrusion occurred near stomata with abaxial cuticles (Figure 85), but the solvent appeared not to have passed through the stomatal pore. Wax rodlets would have been observed within the stomatal antechambers had this occurred and these were free of wax. The closed pore aperture prevented solvent passage (Figure 86). It is apparent that stomatal pores viewed from the underside are blocked by the cutin lining (Figure 87). Thus, isolated cuticle proved to be an ideal substrate for wax deposition and is a better model of the plant system than the ceramic discs and filters used by Jeffree et al. [89].

Environment and Ultrastructure

Temperature, relative humidity and air speed

Environment had less effect upon wax ultrastructure using the wick-feed technique than has been described for whole plant systems where the biosynthetic system is also subject to modification. Effects of environment on blueberry and citrus wax ultrastructure are illustrated in Figures 88 to 93 and 94 to 99, respectively. Temperature in the range studied had little effect upon ultrastructure of both blueberry and

Figures 83-86. Blueberry leaf wax deposited on isolated citrus leaf cuticles using a modified wick-feed technique after Jeffree [88]--effect of cuticle type.

Fig. 83. Large zone of deposition through adaxial cuticle.

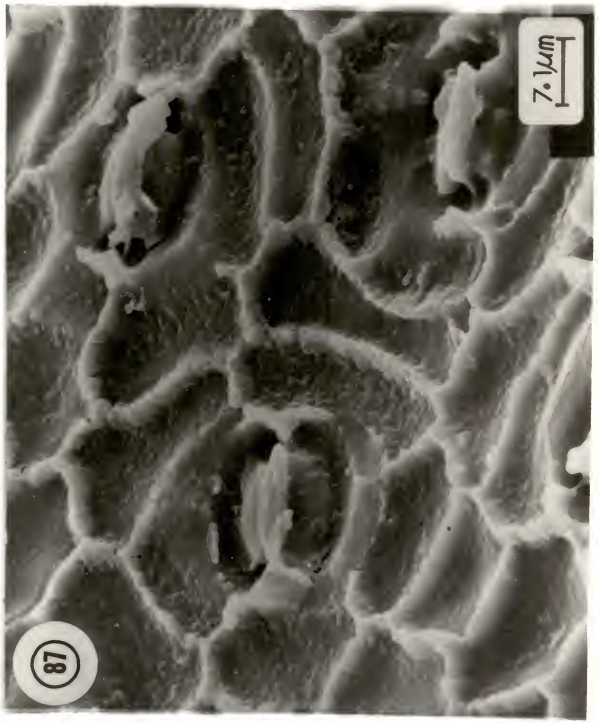
Fig. 84. Scattered zones of deposition through adaxial cuticle.

Fig. 85. Deposition through abaxial cuticles showing proximity to stomata.

Fig. 86. Deposition through abaxial cuticle showing wax-free stomatal pore.

(83)
3·43μm
(84)
0·1mm
(85)
5·6μm
(86)
3·3μm

Figure 87. Underside of citrus leaf cuticle isolated in zinc chloride/hydrochloric acid. (The ridges are cutin that was between the anticlinal cell walls The cuticular lining of the stomatal chambers has joined after extraction to close off the pore.)


87
7.1μm

Figures 88-93. Blueberry leaf wax (March extraction) (0.55 mg/1.5 ml solvent) deposited on isolated citrus leaf cuticles using modified wick-feed technique after Jeffree [88]--effect on ultrastructure of environmental factors during extrusion and deposition.

Fig. 88. 21°C, still air, ambient relative humidity.

Fig. 89. 35°C, still air, ambient relative humidity.

Fig. 90. 20°C, moving air, ambient relative humidity.

Fig. 91. 21°C, still air, low relative humidity (desiccator).

Figs. 92-93. 21°C, still air, high relative humidity (saturated).

(S1)
2.0μm
(S2)
3.6μm
(S3)
1.4μm
(S4)
2.2μm
(S5)
1.8μm
(S6)
0.5μm

Figures 94-99. Leaf waxes from immature (April) and mature (November) 'Pineapple' orange deposited on isolated citrus leaf cuticles using a modified wick-feed technique after Jeffree [88]--effect on ultrastructure of environmental factors during extrusion and deposition--wax concentrations 0.55 mg/1.5 ml solvent.

Fig. 94. Immature-leaf wax, 35°C, still air, ambient relative humidity.

Fig. 95. Mature-leaf wax, 35°C, still air, ambient relative humidity.

Fig. 96. Immature-leaf wax, 21°C, still air, high relative humidity.

Fig. 97. Mature-leaf wax, 21°C, still air, high relative humidity.

Fig. 98. Immature-leaf wax, 21°C, moving air, ambient relative humidity.

Fig. 99. Mature-leaf wax, 21°C, moving air, ambient relative humidity.

(a)
2·4μm
(b)
3·8μm
(c)
2·8μm
(d)
5·8μm
(e)
2·7μm
(f)
1·4μm

citrus waxes. Blueberry wax consisted of well formed rodlets at 21°C typical of natural leaf wax (Figure 88). Rodlets were occasionally shorter and more stubby at 35°C (Figure 89). It is not clear whether this is a temperature effect as similar variations occurred with differences in concentration at the surface. Waxes from immature (Figure 94) and mature (Figure 95) 'Pineapple' orange leaves at 35°C were similar to those at 21°C (not shown). Whole-plant studies have shown a dependence of ultrastructure on both temperature and light [10, 66, 94, 109, 181]. Differences related mostly to the density of wax on the surface and changes in the crystalline form. Baker [10] observed marked changes in morphology by varying light and temperature, while differences in chemistry were found to be minimal. Changes of light intensity were shown to alter chain length of surface waxes [52] without altering the proportion of wax constituents. Temperature has been shown to affect the quantity of wax (equivalent to wax load) deposited on plant surfaces [4, 6, 10, 17, 36, 83, 84]. Wax load was shown to influence the dimensions and distribution of wax structures and the ultrastructure of wax types in an artificial system was shown to be dependent upon the rate of solvent evaporation [20, 28]. Thus, temperature effects on ultrastructure in the whole plant system are a result of interactions among wax loads, rates of crystallizations and probable alterations of chain lengths. Armstrong and Whitecross [6] concluded that variations in wax fine structure, as influenced by growth temperature, resulted from effects at the biochemical level.

Wind had a marked effect upon blueberry wax structure (Figure 90). The moving air caused rapid evaporation of the solvent causing the wax to clump. Structure was poorly developed, the rodlets remaining short,

thick and fused. Platelet formation was reduced with citrus waxes (Figure 98). Amorphous waxes showed the least amount of structural differentiation (Figure 99). Hall and Donaldson [62] observed that abrasion from wind speeds in excess of 30 knots (52 kmh^{-1}) caused up to 50% loss of surface wax of *Trifolium repens*. Apparently no studies relating air speed to wax deposition have been reported.

Blueberry rodlet wax was consistently fine structured at low relative humidity (Figure 91). The rodlets were uniform, long and almost filamentous. Blueberry wax ultrastructure was poorly developed at high relative humidity, being mostly amorphous (Figure 92) with only occasional regions exhibiting rodlet structure (Figure 93). Little or no difference was observed with citrus waxes at low (similar to Figure 94) and high relative humidity (Figures 96, 97). Thus, blueberry wax was more susceptible to alterations in deposition at high humidity than citrus wax. During the experiment, water vapor in the high relative humidity chambers condensed on all surfaces, including the cuticle through which the waxes were extruding. This may be equated with heavy dew or rain. It caused the general loss of rodlet structure similar to that which occurs naturally in the field. Blueberry leaf surfaces lost much of their rodlet ultrastructure by April. This rapid loss was associated with the loss of β-diketones. While the loss of any chemical group was not envisaged in the experiment, it is possible that free water prevented the β-diketones from assuming their usual structural forms.

The lack of response of citrus wax deposition to humidity may be due to the high hydrophobicity [54, 75] of the constituent chemical groups

Citrus waxes were high in alcohols which have been shown to be major contributors to the reduction of cuticular transpiration [57, 75].

Ultraviolet radiation

No changes were detected in any of the wax types as a result of 31 or 72 hours continuous radiation by UV-B at > 290 nm. Qualitative chemical analysis by TLC also indicated that exposure to UV-B radiation did not change the wax constituents. Gingrich [53] exposed cockroaches at 254 nm UV (2.8 watts/m^2) for 1 hour periods over each of 13 days. An increase in cuticle dullness was observed and the proportion of hydrocarbons (C_{17} to C_{25}) increased by as much as 73% over the controls. No effects of UV radiation on blueberry and citrus waxes were detected using the wick-feed technique, but it is possible that changes may occur using whole plant systems. Ultraviolet effects at the biochemical level may influence wax chemistry and ultrastructure.

Wax Chemistry and Ultrastructure

Blueberry fruit wax reproduced using the wick-feed techniques was similar to that which occurred naturally (Figure 100). Another sample, separated by preparative TLC, reconstituted and deposited using the wick-feed technique was reproduced equally as well (Figure 101). β-Diketones deposited using the wick-feed method, had ultrastructure (Figure 102) identical to the parent wax. Blueberry fruit wax minus β-diketone was amorphous with no trace of rodlet structure (Figure 103).

Wax from immature 'Pineapple' orange leaves reproduced by the wick-feed method was similar to that which occurred naturally (Figure 104). There were more of the small platelets, however, which were shown to

Figures 100-103. The effect of some wax constituents on ultrastructure determined by a modified wick-feed technique after Jeffree [88]--all wax or wax constituents concentrations were 0.55 mg/1.5 ml solvent and deposited on isolated citrus leaf cuticles.

Fig. 100. Blueberry fruit wax (extracted April 24).

Fig. 101. Blueberry fruit wax separated by preparative TLC and reconstituted.

Fig. 102. β-Diketone separated from blueberry fruit wax.

Fig. 103. Blueberry fruit wax minus the β-diketone.

(100)
wrfy-1
(101)
wrfy-1
(102)
wrfy-9
(103)
wrfy-1

occur in isolated regions on the leaf surfaces. Separation by preparative TLC and reassembly did not alter this structure (Figure 105). However, addition of β-diketones from the blueberry wax resulted in a transformation of the ultrastructure. Citrus wax structure was indistinguishable from blueberry wax (Figures 106, 107). The contribution of β-diketones to rodlet structure of the surface waxes of barley (*Hordeum vulgare*) was suggested by von Wettstein-Knowles [176, 177, 178]. No rodlet structures were formed when β-diketones were absent, as in the wax of wild type leaves. The length and density or both of the rodlets decreased with reduced quantities of β-diketones in the wax. It was suggested that a critical level of β-diketones may be necessary for rodlet structure [176]. β-Diketones and hydroxy-β-diketones from the leaf sheath wax of barley recrystallized as rodlets, as did β-diketones from leaf waxes of *Festuca glauca*, *Triticum* and *Eucalyptus* species when extruded by the wick-feed method [90].

Since β-diketones were almost exclusively responsible for rodlet structure, other wax forms may in general be determined principally by a single dominant chemical group. Thus, the platelet wax observed in immature citrus-leaf waxes was investigated. These platelets should not be confused with the much larger amorphous plates which form as a result of the surface wax cracking.

Primary and secondary alcohols were removed from the wax of immature 'Pineapple' orange leaves and primary alcohols from mature-leaf wax. Each was extruded using the wick-feed technique. The characteristic platelets were reproduced only from the primary alcohols of the immature-leaf wax (Figures 108-110). The primary alcohol from mature-leaf wax by comparison was amorphous (Figure 111). The basis that primary alcohols

Figures 104-107. The effect of some wax constituents on ultrastructure determined by a modified wick-feed technique after Jeffree [88]--all wax or wax constituents concentrations were 0.55 mg/1.5 ml solvent and deposited on isolated citrus leaf cuticles (continued).

Fig. 104. Wax from immature 'Pineapple' orange leaves.

Fig. 105. Wax from immature 'Pineapple' orange leaves separated by preparative TLC and reconstituted.

Figs. 106-107. Wax from immature 'Pineapple' orange leaves plus ß-diketones as 45% of total wax.

104
5·0μm
105
3·0μm
106
1·5μm
107
2·5μm

Figures 108-111. The effect of some wax constituents on ultrastructure determined by a modified wick-feed technique after Jeffree [88]--all wax or wax constituents concentrations were 0.55 mg/1.5 ml solvent and deposited on isolated citrus leaf cuticles (continued).

Figs. 108-110. Primary alcohols from the wax of immature 'Dancy' tangerine leaves.

Fig. 111. Primary alcohols from the wax of mature 'Dancy' tangerine leaves.

(001)
2·9μm
(100)
1·2μm
(110)
4·6μm
(111)
5·0μm

from immature and mature leaves should differ was not investigated. However, it is believed that the differences may be linked with changes in wax hardness or solubility as previously discussed and is possibly a consequence of chain length and branching. Lundqvist et al. [110] reported that waxes on barley, high in primary alcohol content, existed in the form of lobed plates similar to those reported here. The platelet type of waxes in eucalypts are frequently associated with the presence of large amounts of primary alcohols [65, 67]. In w/o 31 mutant pea, platelet structures on the upper surface are much reduced and the wax contains smaller amounts of primary alcohols compared to normal plants [90]. Amorphous wax forms were produced by the wick-feed method using citrus wax secondary alcohol. This is not in agreement with other studies. The secondary alcohols of *Chamaecyparis lawsoniana*, *Ginko biloba*, *Picea pungens*, *P. sitchensis*, *Chelidonium majus*, *Tropaeolum majus*, *Exochorda racemosa* and *Rhuscotinus atropurpurea* recrystallized as hollow tubes [90]. However, chain length of the secondary alcohol in this study was not determined, as it was in others, and this may influence the ability of any chemical group to form specific structures. Baker [10] suggested that a particular morphological form may depend upon rate of exudation and solution concentrations and Jeffree et al. [90] considered that in certain wax mixtures competition may occur among the constituents for expression of their typical morphologies. This may explain differences in whole wax ultrastructure but does not explain why primary alcohols from young citrus leaves behave differently from those of mature leaves.

The ultrastructure of other wax constituents has not been extensively studied. Chambers et al. [28] studied a range of natural waxes

and constituents by evaporation onto glass. Reproductions were generally poor, although paraffins were shown to grow as flat sheets. These may correspond to the polyhedral plates crystallized from pure n-alkanes [20]. Branched chain alkanes are amorphous [20] but n-alkane mixtures can crystallize in amorphous, needle-like or plate-like forms depending on the rate of crystallization, solvent type and degree of purity of the mixture [20]. Thus, by analogy primary alcohols from mature citrus leaves may be amorphous due to molecular changes, possibly including branching.

Leaf Age and Wax Ultrastructure

Comparisons of waxes from immature and mature citrus and blueberry leaves deposited by the wick-feed method is presented in Figures 112-119. Natural citrus plate waxes are compared with those produced by wick-feed in Figures 120-125.

Waxes from immature leaves of each of the three citrus cultivars were similar, having prominent platelet structure (Figures 112, 114, 116). Platelets were slightly cupped with fringed or lobed margins and were upright. These were not typical of waxes on natural immature leaf surfaces which were generally amorphous. Jeffree et al. [90] similarly reported platelet structures for *Citrus limon* wax extruded by wick-feed, whereas normal leaf surfaces were amorphous.

Waxes of mature citrus leaves resembled the natural structure more closely than those of immature leaves. They were amorphous and formed large flat irregular plates (Figures 113, 115, 117) typical of mature leaf surface ultrastructure.

Figures 112-115. A study of wax ultrastructure from immature and mature citrus and blueberry leaves using a modified wick-feed technique after Jeffree [88]--each wax concentration was 0.55 mg/1.5 ml solvent and the waxes were extruded through isolated citrus leaf cuticles.

Fig. 112. Wax from immature 'Pineapple' orange leaves.

Fig. 113. Wax from mature 'Pineapple' orange leaves.

Fig. 114. Wax from immature 'Dancy' tangerine leaves.

Fig. 115. Wax from mature 'Dancy' tangerine leaves.

(112)
2·8μm
(113)
1·4μm
(114)
1·4μm
(115)
2·8μm

Figures 116-119. A study of wax ultrastructure from immature and mature citrus and blueberry leaves using a modified wick-feed technique after Jeffree [88]--each wax concentration was 0.55 mg/1.5 ml solvent and the waxes were extruded through isolated citrus leaf cuticles (continued).

Fig. 116. Wax from immature navel orange leaves.

Fig. 117. Wax from mature navel orange leaves.

Fig. 118. Wax from immature blueberry leaves.

Fig. 119. Wax from mature blueberry leaves.

(116)
5·6μm
(117)
1·4μm
(118)
5·7μm
(119)
2·8μm

Figures 120-125. A comparison of platelet wax ultrastructure between natural and wick-feed surfaces with isolated citrus leaf cuticles.

Fig. 120. Wax from immature navel orange leaves extruded through isolated citrus cuticle (0.55 mg/1.5 ml solvent).

Fig. 121. Wax from immature 'Dancy' tangerine leaves extruded through isolated citrus cuticle (0.55 mg/1.5 ml solvent).

Fig. 122. Surface of 'Pineapple' orange fruit sampled August 10.

Fig. 123. Surface of 'Dancy' tangerine leaf sampled June 28.

Fig. 124. Surface of 'Eureka' lemon leaf sampled November 15.

Fig. 125. Surface of navel orange fruit sampled November 20.

(20)
2.4μm
(21)
4.7μm
(22)
4.1μm
(23)
4.2μm
(24)
2.4μm
(25)
4.4μm

Waxes from both immature (Figure 118) and mature (Figure 119) blueberry leaves were reproduced in forms closely resembling those on the natural leaf surfaces. The loss of rodlet structure in mature-leaf wax can be attributed to the virtual lack of β-diketones.

The development of platelets by wick-feed in wax of immature citrus leaves was not completely anomalous as has been suggested [90]. A close study of surface waxes in developing citrus leaves and fruit revealed that similar structures do occur naturally. Platelets produced by wick-feed are shown in Figures 120 and 121. In Figure 121 it appears as though the platelets developed from, or were extruded through, an underlying layer of amorphous wax. The naturally occurring platelets varied in shape and distribution (Figures 122-125). Those most closely resembling the wick-feed type were observed on 'Pineapple' orange fruit surface (Figure 122). The platelets were mostly associated with dense areas of underlying wax (Figure 123) but were occasionally found in sparser areas (Figure 124). Platelet development was not always complete (Figure 125) in some areas. Platelet structures similar to these were observed on 'Valencia' orange fruit surfaces [50] but were induced by polishing. The potential to produce these platelets naturally, therefore, exists. The reasons they dominate the wax ultrastructure when using the wick-feed methods are not clear. These platelets were shown to be essentially primary alcohols. Studies involving solvents, rates of evaporation and plant and environmental factors are required to clarify this.

Wax Extrusions: Mechanisms

The mechanism of wax extrusion, deposition and chemical differentiation has yet to be resolved. The wick-feed technique [88] provides a useful tool for detailed study. Use of plant cuticles as a substrate makes possible studies of extrusion under more natural conditions.

Wax transport through cuticles

The reproducibility of natural surface ultrastructure achieved using the wick-feed technique supports the concept that waxes are transported in solution [10, 38]. Upon contact with the air, the solvent evaporates causing solidification or crystallization of the wax. Chloroform was used exclusively as the wax solvent in the studies reported here. Jeffree et al. [89] also used chloroform extensively and reported that wax ultrastructure was independent of most organic solvents having dielectric constants in the range 1.8 to 5.0 and boiling points below about 80°C. Chambers et al. [28] similarly concluded that waxes are transported in solution and that the solvent may be the oil characteristic for the plant. Thirteen volatile substances were derived from the epicuticular wax of cranberry (*Vaccinium macrocarpon*) [34]. These were attributed to be responsible for characteristic wax odors while the presence of benzoic acid and other benzenoic and triterpenoid compounds was interpreted as having a defensive role. A wide range of volatile organic compounds were identified in *Ribes nigrum* [134, 135] and vegetables [91, 92] and it is possible that these may constitute a solvent system for wax transport. Current evidence suggests it is unlikely that waxes pass through the cuticle in the form of mobile

precursors which oxidize or polymerize at the surface [66, 178] or are extruded as a softened paste [120, 148, 149]. Natural solvents which may transport wax through the cuticle have not been isolated. Demonstration of a solvent system would be sufficient proof that waxes do travel in solution and the isolation and identification of such a solvent should be an important area for future study.

Pathway for wax transport through cuticles

Electron micrographs of plant cuticles indicate that they can be divided into two main groups, lamellate and non-lamellate. Lamellate cuticles show characteristic lamellae mostly toward the outer cuticle surface and it has been proposed that these are wax lamellae, representing the pathway of wax or its precursors through the cuticle [33, 64, 68, 95]. Plants with lamellate cuticles include *Eucalyptus* species [64, 66], *Prosopis* species [84], *Libertia elegans* [145], *Brassica* and *Musa* spp. [148], *Eragrostis curvula* [109], *Phormium tenax* [86], *Pisum sativum* [93], *Picea sitchensis* [87] and a range of other species [14]. Plants with non-lamellate cuticles include *Trifolium repens*, *Poa colensoi*, *Malus domestica* [62], *Eucalyptus urnigera* [60, 61] and *Plantago major* [44]. In each of these, pores were detected in the cuticle and interpreted as being wax microchannels [44, 60, 61]. No pores have been detected in lamellate cuticles. *Citrus* cuticles are non-lamellate (Albrigo, personal communication) and no evidence of pores has been published. Minute radial canals were observed, however, in *Citrus sinensis* fruit cuticle [160, 162]. Use of the wick-feed technique by Jeffree et al. [89] did support the pore theory, as waxes recrystallized through a porous membrane. They did demonstrate, however, that pore

size and density had no influence upon ultrastructure, a point made by von Wettstein-Knowles [178]. It was not determined whether citrus cuticles used in the wick-feed experiments contained pores. Reproducibility of many different wax surfaces on porous discs [88, 89, 90] and blueberry wax on citrus cuticle suggests that cuticle structure bears little relationship to wax ultrastructure.

It is proposed that the cuticle is an inert medium for wax transport and the nature of the pathways, whether they be pores, lamellae or otherwise, is a function of organization of the cutin matrix which is probably species specific. Jeffree et al. [90] proposed that wax transport occurs in solution by molecular diffusion via the intermolecular spaces in the membrane. If this were true then wax solutions should traverse lamellate and non-lamellate cuticles with equal ease with equal reproducibility of ultrastructure. This may now be simply tested using a variety of cuticle and wax types with the wick-feed technique.

This technique was used to determine the influence of embedded waxes upon the passage of surface waxes. Wax deposition was compared using surface dewaxed cuticles and totally dewaxed cuticles. No differences were detected as shown in Figures 126, 127 and 128. Differences observed were due to variable wax accumulation. Inverting the cuticles occasionally resulted in wax deposition in the periclinal zones (Figure 126) but this was not typical and deposition occurred in most cases along the anticlinal ridges (Figures 127-129). The lack of rodlets in the wax extruded through an inverted, totally dewaxed cuticle (Figure 129) was due to the high humidity, > 95%, during extrusion. It is believed that wax deposition on the anticlinal ridges is more likely to be a simple "wick" effect, whereby the solvent having passed through the

Figures 126-131. The effect of embedded waxes in isolated cuticles on wax depositions (wick-feed) and a consideration of rodlet extrusion.

Fig. 126. Wax from immature blueberry leaves (0.55 mg/1.5 ml solvent) extruded through an inverted, surface dewaxed, isolated citrus cuticle.

Fig. 127. Wax from immature blueberry leaves (0.55 mg/1.5 ml solvent) extruded through an inverted, totally dewaxed, isolated citrus cuticle.

Fig. 128. Wax from immature blueberry leaves (0.55 mg/1.5 ml solvent) extruded through an inverted, surface dewaxed, isolated citrus cuticle.

Fig. 129. Wax from immature blueberry leaves (0.55 mg/1.5 ml solvent) extruded at high relative humidity through an inverted, totally dewaxed, isolated citrus cuticle.

Fig. 130. Rodlet structure on the surface of mature blueberry fruit.

Fig. 131. Rodlet extrusion from cracks in the cuticle of mature blueberry leaves.

(120)
6.4μm
(121)
4.7μm
(128)
6.4μm
(129)
7.4μm
(130)
0.6μm
(131)
7.0μm
(132)
7.0μm

cuticle, travelled to the highest point, rather than an indication of preferential transport sites.

Blueberry wax rodlets *in vivo* (Figure 130) appear as if they had emerged from pores. Variable thickness of the rodlets, however, gives no indication of possible pore size, the narrowest rodlets being approximately 300 nm in diameter. Pores this size would be visible and, hence, much larger than the 40 nm pores observed by Hall [60, 61].

Reduction in wax flow with increasing leaf age was suggested to be due to a final hardening of the primary cuticle [148]. Davis [38] suggested that pores did not exist beyond a critical stage of growth. The data indicated reduced wax synthesis after full leaf expansion and some chemical groups were being deposited in preference to others. In senescent blueberry leaves, triterpenoid production continued after other chemical groups had stopped accumulating. The reason for this is not clear and it is possible that recording changes in surface wax does not truly reflect synthesis reactions which occur in the epidermis. Should the pathways through the cuticle become restricted in mature leaves the surface wax chemistry would then reflect the ease of passage of one chemical group over another rather than reflecting biosynthetic changes. This hypothesis is supported by the observations of near-senescent blueberry leaves (Figure 131). Cracks had developed in the cuticle and fine rodlet wax had emerged all along these. This was probably β-diketone which had reached the surface through the path of least resistance, the normal cuticular pathway probably being blocked as a result of ageing. Since the embedded waxes had no influence on wax flow in either direction through the cuticle, and they are retained within the matrix *in vivo*, it is proprosed that surface waxes by-pass these en route to

the surface. Thus, the embedded waxes may be bound within the matrix. The following lends support to this hypothesis. Polar pores were demonstrated in the polymer matrix of isolated citrus leaf cuticles [150, 156]. These were 0.46 to 0.55 nm in diameter, continuous across the cutin membrane [150] and composed of clusters of carboxyl (COOH) groups. COOH groups are donated by residues of acidic amino acids, polygalacturonic acid and non-esterified COOH groups of the cutin polymer [155, 156]. Only those pores lined with relatively strong acidic groups conduct water and small solutes at low pH [150]. With increasing pH, new pores lined with COOH groups of lower acid strength are postulated to come into being [150]. Extraction of cuticular waxes from isolated membranes increased their water permeability by a factor of 300 to 500 [151]. By treating the cutin matrix and cuticular waxes as two resistances in series, it was shown that water permeability of cuticles was completely determined by the waxes [151]. No differentiation was made, however, between surface and embedded waxes and the extraction procedure removed both. Removal of the waxes caused such a large increase in water permeability and this has been attributed to COOH-lined polar channels in the matrix; thus, it is proposed that the embedded waxes, mostly C_{16}, C_{18} fatty acids, are bound to the cutin matrix preventing the exposure of COOH groups. The proposed binding may also explain why waxes remain embedded and require more stringent solvent extraction to remove. Repeating Schonherr's [151] experiments with progressively dewaxed isolated cuticles may better determine the relative importance of surface and embedded waxes in affecting water permeability. This would also test the hypothesis that embedded waxes are bound to COOH groups in the cutin matrix.

Structural development of the surface wax complex

A major advantage of the wick-feed method is the ability to vary wax load in order to study morphological development of wax ultrastructure. Several explanations have been offered to account for the development of wax rodlets. Hall [60] proposed that the wax exuded from pores in a manner similar to lava from a volcano. This does not account for the fine structures observed in most rodlet wax types nor the branching of these. Hallam [66] proposed that the wax rodlets on eucalypts were hollow and formed by the rolling of narrow plates. Rodlet or filamentous structures of *Eucalyptus globulus* [68] are multi-branched and Hallam and Juniper [68] proposed that these were formed by successive growths from within the structures. von Wettstein-Knowles [178] suggested that wax was extruded through pores onto the cuticle surface and continuous exudation of new wax pushes the initial wax away, leaving the most recent at the surface. This is difficult to reconcile with the view of the wick-feed studies of Jeffree et al. [89] and those reported here. Close examination of many micrographs showing rodlet development in blueberry wax has led to the following hypothesis: Initial wax deposited at the surface forms a small mound (Figure 132(1)). Continual secretion causes a saturated wax solution film to be present at the surface at all times. The rodlet first appears as a small "button" from this mound (Figure 132(2)). The saturated solvent flows upwards to the highest point before evaporating. This would be a result of surface tension and capillary effects. Evaporation at the tip causes the "button" to elongate to a rudimentary rodlet (Figure 128(3)). This could be likened to the formation of stalactites, but

in reverse. Any slight anomaly, uneven flow or molecular branching in the crystal structure would cause a slight buildup on the side of the rodlet (Figure 132(4)). This would provide a new evaporating surface and solvent flow or surface creep would be divided (Figure 132(5)) eventually to form a branched rodlet (Figure 132(6)). Each of these stages has been observed *in vivo* and *in vitro* using the wick-feed technique. Hollow tube waxes may develop similarly. Platelet waxes, such as those described for waxes from immature citrus leaves, may form in a similar manner. The crystal shape is a function of chemistry but it is suggested that growth occurs from the extremities due to solvent evaporation from the upper edges.

It is not clear whether new wax produced where large deposits already exist is deposited below or above the existing wax. In citrus fruits in which wax synthesis continued through to maturity it appeared that the new wax extruded through the existing wax when the fruits were immature. However, as the fruit matured and the wax layers thickened, these thick layers may have prevented the solvent from reaching the surface and slow evaporation may have occurred in the underlying wax layers. This would explain the suspected chemical heterogeneity of the wax, as the new wax, richer in aldehydes and fatty acids, would accumulate under the paraffins and alcohols. Lifting and cracking of the surface wax layers of citrus leaves and fruits may be at least partially due to pressures from underlying new wax. Solvent action at the interface of old and newly extruded wax may also be responsible. Blocking of solvent-wax solution by old wax could cause a tunnelling effect under the old wax until a release point occurred. This may also account for heterogeneity and may explain the concentration of wax deposits in some

areas as observed. For example, platelet wax, believed to be principally primary alcohol, was usually seen on citrus leaf and fruit surfaces in concentrated regions. This explanation does not account for those wax forms which are often organized into linear, concentric or radial arrangements [90]. These do not occur using the wick-feed method and their occurrence has not been explained. Jeffree et al. [90] considered that they may be a function of the cuticular membrane, while Chambers et al. [28] discussed the possibility of epitaxy.

Continual wax synthesis and transfer in solution to the cuticle surface would imply a continuous liquid phase between the epidermal cells and the cuticle surface underlying existing wax. This would facilitate the reentry of wax components back to the epidermis and also chemical interaction between groups on the surface. This then supports the hypothesis of Cassagne and Lessire [24] as described previously. In conclusion, therefore, complex and heterogeneous epicuticular wax can be considered to be dynamic with its ultrastructure being a function of chemistry, environment and plant physiological maturity.

Figure 132. Schematic diagram showing the proposed mechanism for the formation of branched rodlets.

(1) initial wax mound

(2) "button" formation as preformer of rodlets

(3) rudimentary rodlet

(4) rodlet extension and branching point

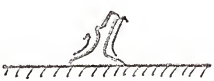
(5) rodlet growth with branching

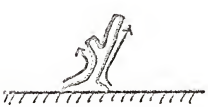
(6) fully developed branched rodlet

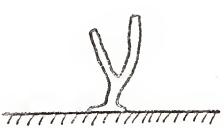
(1) 

(2) 

(3) 

(4) 

(5) 

(6) 

APPENDIX

Table A-1. Wax constituents of 'Pineapple' orange leaves: Changes in percentage of total wax, quantity per leaf and per unit leaf area.

	March 24			April 8			May 12			June 23		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	3.6 (0.7)[2]	40.6	1.6	12.3 (1.3)	33.1	0.9	5.0 (0.8)	29.1	0.7	17.2 (2.6)	157.6	4.4
Esters	---	---	---	---	---	---	---	---	---	---	---	---
Ketones	---	---	---	---	---	---	---	---	---	11.0 (2.6)	107.2	2.8
Aldehydes	---	---	---	6.4 (2.9)	17.2	0.5	9.2 (1.3)	46.2	1.1	10.2 (0.8)	99.4	2.6
Secondary alcohols	86.9 (2.0)	979.2	38.7	3.9 (1.4)	10.6	0.3	---	---	---	---	---	---
Triterpenyl acetate	---	---	---	---	---	---	8.4 (0.3)	42.2	1.0	13.5 (1.8)	131.6	3.4
Primary alcohols	5.7 (1.0)	64.3	2.5	54.8 (10.6)	148.7	4.3	38.6 (1.1)	194.1	4.9	29.8 (5.6)	290.5	7.6
Fatty acids	3.8 (1.0)	42.8	1.7	17.4 (2.7)	46.8	1.3	25.1 (5.2)	126.2	3.2	10.7 (0.9)	104.3	2.7
Acidic triterpenoid	---	---	---	5.2 (0.3)	14.0	0.4	12.9 (4.8)	64.8	1.6	7.5 (1.5)	73.1	1.9
Means		1126.1 (639)	52.9 (31.4)		269.3 (126.3)	7.3 (3.4)		502.8 (202.6)	13.4 (2.3)		974.9 (167.7)	25.5 (5.4)
Mean surface area (cm²)	25.3 (16.2)			34.3 (17.6)			39.6 (19.5)			38.2 (14.0)		

[2](± S.E.).

Table A-1. Continued.

	August 10			September 27			November 16			December 16		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	16.1 (1.4)[z]	134.7	3.3	8.8 (0.3)	67.2	1.9	14.5 (3.7)	115.2	3.1	7.2 (1.5)	55.1	1.5
Esters	---	---	---	27.7 (1.3)	211.7	6.1	---	---	---	---	---	---
Ketones	---	---	---	---	---	---	---	---	---	0.3 (0.7)	2.3	0.06
Aldehydes	13.5 (3.7)	112.9	2.7	5.2 (0.3)	39.7	1.1	10.0 (3.0)	78.9	2.1	5.3 (0.0)	40.6	1.1
Secondary alcohols	---	---	---	---	---	---	---	---	---	0.0 (0.3)	0.1	0.1
Triterpenyl acetate	12.9 (0.7)	107.9	2.6	11.6 (1.3)	88.6	2.5	14.8 (1.4)	116.9	3.1	12.4 (0.7)	95.0	2.6
Primary alcohols	30.0 (2.5)	250.9	6.1	27.8 (2.7)	212.5	6.1	43.1 (6.4)	340.3	9.2	51.0 (7.1)	390.7	10.6
Fatty acids	14.8 (2.5)	123.8	3.0	11.2 (1.9)	85.6	2.5	8.3 (1.0)	65.5	1.8	10.2 (4.2)	78.1	2.1
Acidic Triterpenoid	12.7 (2.4)	106.2	2.6	7.7 (1.3)	58.8	1.7	9.2 (0.1)	72.6	1.9	12.8 (0.2)	98.1	2.7
Means		836.5 (244.6)	20.6 (1.1)		764.4 (275.1)	21.9 (0.7)		789.7 (217.5)	21.7 (1.0)		768.2 (2.3)	21.2 (0.1)
Mean surface area (cm²)	40.8 (14.0)			36.6 (11.4)			38.8 (11.4)			36.1 (0.3)		

[z](± S.E.)

Table A-2. Wax constituents of 'Dancy' tangerine leaves: Changes in percentage of total wax, quantity per leaf and per unit leaf area.

	March 24			April 8			May 12			June 28		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	1.7 (0.0)[2]	4.1	0.4	13.3 (0.9)	35.0	1.7	24.0 (1.0)	170.1	7.6	34.4 (1.6)	355.0	14.0
Esters	---	---	---	---	---	---	---	---	---	---	---	---
Ketones	0.4 (0.0)	0.9	0.1	---	---	---	10.1 (2.2)	69.3	3.1	11.3 (0.3)	130.0	4.6
Aldehydes	4.3 (0.0)	10.4	1.0	1.0 (0.5)	2.6	0.1	1.9 (0.3)	13.0	0.6	7.3 (1.4)	84.0	3.0
Secondary alcohols	56.3 (3.2)	136.5	12.9	4.7 (0.6)	12.4	0.6	8.9 (4.3)	61.1	2.7	9.8 (0.6)	112.8	4.0
Triterpenyl acetate	2.2 (2.2)	5.3	0.5	5.3 (2.7)	13.9	0.7	4.8 (3.6)	32.9	1.4	2.0 (0.1)	23.0	0.8
Primary alcohols	26.0 (1.5)	63.2	6.2	37.7 (1.0)	99.2	4.9	29.4 (0.2)	201.7	8.9	13.8 (3.7)	159.3	5.6
Fatty acids	6.3 (2.1)	15.3	1.4	33.5 (1.8)	88.2	4.4	16.5 (4.4)	113.2	5.0	14.0 (0.3)	161.1	5.7
Acyclic triterpenoid	1.9 (0.8)	4.6	0.4	4.5 (0.8)	11.8	0.6	3.6 (2.5)	24.7	1.1	7.3 (0.6)	84.0	2.9
Means		242.7 (59.3)	23.1 (12.1)		263.3 (50.5)	13.1 (9.7)		686.1 (368.1)	30.5 (4.2)		1150.7 (732.2)	40.6 (11.6)
Mean surface area (cm^2)		12.3 (6.1)			25.6 (11.8)			22.9 (12.0)			27.0 (3.0)	

[2](± S.E.)

Table A-2. Continued.

	August 10			September 27			November 15			December 15		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	24.8 (0.6)[1]	232.5	7.7	25.1 (3.2)	---	---	17.0 (0.3)	150.9	4.3	13.6 (2.2)	116.7	3.6
Esters	---	---	---	---	---	---	---	---	---	---	---	---
Ketones	1.0 (0.3)	9.4	0.3	1.7 (0.2)	---	---	---	---	---	0.8 (0.2)	6.8	0.2
Aldehydes	6.6 (0.1)	61.9	2.0	7.0 (0.1)	---	---	6.9 (1.6)	60.4	1.7	8.7 (2.6)	74.0	2.3
Secondary alcohols	3.8 (1.0)	35.6	1.2	10.3 (4.6)	---	---	13.1 (3.6)	111.1	3.2	1.0 (0.6)	8.5	0.2
Triterpenyl acetate	2.1 (3.0)	19.7	0.6	---	---	---	---	---	---	10.7 (1.1)	91.0	2.8
Primary alcohols	34.5 (3.7)	324.4	10.7	37.4 (5.7)	---	---	45.1 (0.4)	382.3	10.9	44.2 (3.3)	376.0	11.7
Fatty acids	16.8 (1.8)	157.5	5.2	7.5 (0.6)	---	---	7.6 (0.3)	64.4	1.8	12.1 (1.0)	102.9	3.2
Acidic triterpenoid	10.4 (1.1)	97.5	3.2	10.0 (4.1)	---	---	9.5 (2.4)	80.5	2.3	8.9 (4.4)	75.7	2.3
Means		937.6 (30.6)	31.0 (0.1)		---	---		847.8 (112.5)	24.3 (1.3)		850.6	26.5
Mean surface area (cm^2)	30.1 (0.3)			29.2			34.8 (2.7)			32.0		

[1](± S.E.).

Table A-3. Wax constituents of navel orange leaves: Changes in percentage of total wax, quantity per leaf and per unit leaf area.

	March 26			April 10			May 15			June 8		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	3.6 (0.3)[2]	8.1	1.0	4.2 (2.3)	12.3	0.3	17.9 (2.4)	130.9	4.8	17.0 (4.8)	354.4	4.6
Esters	0.4 (0.3)	0.9	0.1	0.7 (0.3)	2.0	0.05	1.0 (0.0)	7.8	0.2	1.6 (0.5)	33.3	0.4
Ketones	3.8 (0.8)	8.7	1.1	2.9 (0.6)	8.5	0.2	18.6 (2.2)	145.4	4.9	1.6 (0.5)	33.3	0.4
Aldehydes	0.9 (0.4)	2.0	0.2	1.8 (0.2)	5.3	0.1	2.2 (0.4)	17.2	0.6	4.7 (0.2)	98.0	1.3
Secondary alcohols	67.8 (1.0)	152.3	19.8	74.1 (5.4)	217.8	5.3	11.7 (1.5)	91.4	3.1	1.5 (0.4)	31.3	0.4
Triterpenyl acetate	0.9 (0.0)	2.0	0.2	0.8 (0.1)	2.3	0.05	5.6 (1.2)	43.7	1.6	8.0 (0.6)	166.8	2.2
Primary alcohols	16.2 (0.2)	36.3	4.7	7.6 (1.3)	22.3	0.5	23.1 (0.3)	180.5	6.2	55.2 (4.6)	1150.9	15.0
Fatty acids	2.0 (1.2)	4.5	0.6	3.9 (2.5)	11.4	0.3	11.7 (2.4)	91.4	3.1	3.7 (1.0)	77.1	1.0
Acidic triterpenoid	4.3 (0.3)	9.6	1.2	4.0 (1.4)	11.7	0.3	0.2 (2.7)	64.7	2.2	6.7 (1.5)	139.7	1.8
Means		224.4 (65.6)	29.2 (1.7)		293.2 (55.4)	7.3 (1.1)		701.6 (557.3)	25.5 (5.1)		2085.0	27.2
Mean surface area (cm²)		7.7 (2.7)			40.6 (13.3)			29.2 (15.7)			76.5	

[2](± S.E.).

Table A-3. Continued.

	July 10			August 21			October 6			November 20		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	29.8 (1.0)[2]	640.2	9.0	14.4 (0.1)	143.5	2.8	16.5 (0.5)	131.0	2.8	11.4 (4.2)	81.2	1.9
Esters	1.6 (0.1)	34.8	0.5	0.7 (0.1)	6.9	0.1	1.0 (0.3)	8.0	0.1	0.4 (0.5)	2.8	0.1
Ketones	1.7 (0.5)	36.9	0.5	2.5 (0.4)	20.9	0.4	---	---	---	0.2 (0.5)	1.4	0.03
Aldehydes	7.7 (2.2)	167.5	2.3	7.2 (1.7)	71.7	1.4	10.0 (0.6)	79.9	1.7	6.9 (0.3)	49.1	1.2
Secondary alcohols	---	---	---	---	---	---	---	---	---	---	---	---
Triterpenyl acetate	9.7 (5.1)	211.0	2.9	18.0 (0.7)	179.4	3.5	12.3 (2.0)	98.2	2.1	10.9 (4.0)	77.7	1.8
Primary alcohols	33.3 (8.0)	724.4	10.1	47.2 (1.7)	474.4	9.3	37.9 (3.5)	302.8	6.5	39.5 (6.3)	280.7	6.7
Fatty acids	5.1 (1.9)	110.9	1.5	6.0 (0.8)	59.8	1.2	10.3 (2.5)	82.3	1.7	16.0 (1.1)	114.0	2.7
Acidic triterpenoid	11.1 (3.2)	241.4	3.4	4.0 (0.2)	39.8	0.8	12.0 (0.6)	95.9	2.0	14.7 (5.6)	104.7	2.5
Means		2175.4	30.4		996.7	19.4		799.0	17.0		712.5 (6.3)	17.0 (0.2)
Mean surface area (cm²)		71.5			51.2			46.8			41.7 (1.2)	

[2](± S.E.).

Table A-4. Wax constituents of 'Eureka' lemon leaves: Changes in percentage of total wax, quantity per leaf and per unit leaf area.

	April 9			May 12			June 28			August 10		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	30.0 (0.2)[z]	60.5	2.3	27.4 (4.1)	155.8	3.6	57.2 (0.2)	900.4	25.6	31.1 (6.2)	376.3	6.9
Esters	1.7 (0.0)	3.4	0.1	4.0 (0.1)	22.7	0.5	2.4 (1.6)	35.0	0.9	3.2 (0.4)	38.6	0.7
Ketones	---	---	---	---	---	---	2.5 (1.0)	36.4	0.9	0.7 (0.0)	8.4	0.1
Aldehydes	3.2 (0.1)	6.4	0.2	7.8 (6.5)	44.3	1.0	5.2 (2.5)	75.8	2.0	8.8 (3.2)	106.1	1.9
Secondary alcohols	2.8 (1.8)	5.6	0.2	2.1 (0.9)	11.9	0.3	1.5 (1.0)	21.9	0.6	1.8 (0.6)	21.7	0.4
Triterpenyl acetate	10.2 (1.0)	20.7	1.4	1.4 (0.1)	7.9	0.2	1.9 (0.8)	27.7	0.7	7.2 (0.2)	86.8	1.6
Primary alcohols	27.4 (0.4)	55.3	2.1	51.9 (0.1)	295.2	6.8	12.6 (0.0)	183.8	4.8	27.6 (2.5)	332.8	6.1
Fatty acids	9.9 (3.5)	19.9	0.7	5.4 (1.6)	30.7	0.7	5.9 (2.7)	86.1	2.2	14.4 (1.1)	173.6	3.2
Acidic triterpenoid	6.8 (0.2)	13.7	0.5	---	---	---	0.8 (0.0)	11.7	0.3	5.2 (1.6)	62.7	1.1
Means		201.8 (10.4)	0.0 (1.8)		568.8 (179.0)	13.2 (0.6)		1459.0	38.1		1208.0	22.1
Mean surface area (cm^2)		26.2 (7.4)			43.3 (15.4)			38.3			54.5	

[z](± S.E.).

Table A-4. Continued.

	September 27			November 15			December 15		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	10.6 (1.4)[z]	107.4	2.6	6.6 (0.9)	91.9	1.5	12.3 (2.2)	98.9	1.9
Esters	38.2 (6.2)	387.2	9.3	26.0 (0.7)	361.9	5.8	2.3 (1.2)	18.5	0.3
Ketones	0.2 (0.2)	2.0	0.04	2.0 (1.1)	27.8	0.4	3.2 (2.0)	25.7	0.5
Aldehydes	6.4 (2.5)	64.8	1.5	9.3 (3.1)	129.4	2.1	11.7 (0.0)	94.1	1.8
Secondary alcohols	3.2 (0.5)	32.4	0.8	3.8 (1.2)	52.9	0.8	4.0 (0.9)	32.2	0.6
Triterpenyl acetate	5.1 (1.0)	51.0	1.2	8.9 (2.4)	123.9	2.0	7.1 (0.6)	57.1	1.1
Primary alcohols	24.9 (1.0)	252.4	6.1	21.5 (0.3)	299.3	4.8	35.5 (3.4)	285.5	5.6
Fatty acids	9.9 (0.9)	100.3	2.4	12.3 (1.7)	171.2	2.7	15.5 (2.9)	124.7	2.5
Acidic triterpenoid	1.5 (1.0)	15.2	0.4	9.6 (1.3)	133.6	2.1	8.5 (0.7)	68.4	1.3
Means		1013.7	24.3		1392.1 (232.5)	22.2 (2.1)		804.4 (119.6)	15.9 (1.6)
Mean surface area (cm^2)		41.7			62.5 (4.4)			50.6 (2.5)	

[z](± S.E.).

Table A-5. Wax constituents of 'Eureka' lemon summer flush leaves: Changes in percentage of total wax, quantity per leaf and per unit leaf area.

	June 20			August 10			September 27			November 15		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	44.2 (3.2)[z]	782.8	13.3	29.7 (0.5)	176.8	3.6	15.3 (0.4)	107.5	2.1	23.5 (4.8)	140.8	2.3
Esters	15.2 (2.6)	269.2	4.5	1.7 (0.5)	10.1	0.2	25.9 (0.7)	182.0	3.6	5.2 (0.0)	32.9	0.5
Ketones	0.6 (0.1)	10.6	0.2	1.0 (0.4)	5.9	0.1	1.4 (0.1)	9.8	0.2	---	---	---
Aldehydes	4.5 (1.5)	79.7	1.3	10.8 (2.2)	64.5	1.3	2.3 (0.3)	16.1	0.3	12.0 (0.8)	76.0	1.1
Secondary alcohols	9.5 (1.1)	168.2	2.8	2.0 (1.4)	11.9	0.2	1.6 (1.3)	11.2	0.2	1.0 (1.3)	6.3	0.1
Triterpenyl acetate	5.1 (1.2)	90.3	1.5	9.9 (2.8)	59.1	1.2	11.9 (4.1)	83.6	1.6	14.0 (3.2)	88.7	1.3
Primary alcohols	8.4 (0.3)	148.8	2.5	22.2 (2.7)	132.6	2.7	21.3 (4.6)	149.7	2.9	19.8 (0.3)	125.4	1.9
Fatty acids	6.3 (3.4)	111.6	1.9	15.4 (0.3)	92.0	1.9	14.4 (0.9)	101.2	2.0	13.9 (3.7)	88.0	1.3
Acidic triterpenoid	6.2 (0.7)	109.8	1.9	7.3 (1.1)	43.6	0.9	5.9 (1.1)	41.4	0.8	10.6 (2.0)	67.1	1.0
Means		1771.1 (1026.0)	29.9 (7.1)		597.5	12.3		702.7	13.9		633.5 (3.7)	9.7 (0.6)
Mean surface area (cm²)		59.3 (21.0)			43.4			50.3			66.1 (3.4)	

[z](± S.E.).

Table A-6. Wax constituents of 'Pineapple' orange fruit: Changes in percentage of total wax, quantity per fruit and per unit fruit surface area.

	April 22			May 12			June 28			August 10		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	34.6 (1.8)[2]	29.7	9.9	43.7 (1.7)	311.2	21.3	33.7 (5.5)	838.2	14.0	16.3 (4.2)	614.7	5.0
Esters	---	---	---	11.9 (1.2)	84.7	5.8	2.7 (2.4)	67.1	1.1	---	---	---
Ketones	16.1 (1.3)	13.8	4.6	4.2 (4.3)	29.9	2.0	14.2 (8.6)	353.2	5.9	1.9 (0.2)	60.0	0.6
Aldehydes	---	---	---	0.4 (0.1)	59.8	4.1	10.5 (3.6)	261.1	4.3	33.8 (3.7)	1225.3	11.2
Secondary alcohols	10.2 (0.9)	8.8	2.9	4.5 (0.5)	32.0	2.2	5.1 (3.9)	151.7	2.5	9.1 (1.3)	237.4	2.8
Triterpenyl acetate	---	---	---	3.6 (1.2)	25.5	1.7	---	---	---	---	---	---
Primary alcohols	29.1 (0.8)	24.9	8.3	11.7 (0.3)	83.3	5.7	5.5 (1.8)	164.1	2.7	16.1 (3.4)	508.4	4.9
Fatty acids	9.9 (3.0)	8.5	2.8	8.5 (5.1)	60.5	4.1	17.3 (6.3)	430.3	7.2	10.3 (1.0)	325.3	3.2
Acidic triterpenoid	---	---	---	3.5 (1.0)	24.9	1.7	8.8 (2.7)	218.9	3.6	7.5 (2.5)	236.8	2.3
Means		85.8 (28.7)	28.3 (3.3)		712.1 (82.1)	52.2 (16.7)		2487.3 (29.2)	40.9 (6.8)		3153.0	30.7
Mean surface area (cm²)		3.0 (1.3)			14.0 (4.0)			60.8 (10.9)			102.6	

[2](± S.E.).

Table A-6. Continued.

	September 27			November 15			December 15		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	6.3 (2.2)[z]	436.3	3.1	13.2 (6.5)	1795.6	10.9	5.7 (0.7)	771.1	4.4
Esters	5.8 (0.8)	408.6	2.9	1.5 (0.8)	204.0	1.2	6.7 (0.9)	906.4	5.2
Ketones	---	---	---	1.6 (0.0)	217.6	1.3	---	---	---
Aldehydes	40.0 (7.1)	2770.2	20.0	41.7 (14.3)	5686.0	34.6	39.4 (1.1)	5330.4	30.8
Secondary alcohols	24.0 (1.4)	1662.1	12.0	2.4 (1.1)	326.4	2.0	2.8 (1.4)	378.8	2.2
Triterpenyl acetate	---	---	---	13.6 (3.4)	1850.0	11.3	10.4 (0.3)	1407.0	8.1
Primary alcohols	6.8 (1.1)	470.9	3.4	4.3 (0.9)	584.9	3.6	3.4 (1.1)	459.9	2.6
Fatty acids	9.3 (6.1)	644.0	4.6	14.1 (0.1)	1918.0	11.7	24.9 (1.6)	3368.7	19.4
Acidic triterpenoid	7.8 (1.8)	540.2	3.9	7.6 (1.5)	1033.8	6.3	6.7 (1.5)	906.4	5.2
Means		6925.5 (283.6)	50.1 (0.1)		13603.8 (18.2)	83.1 (7.3)		13523.2 (229.8)	78.2 (2.9)
Mean surface area (cm²)		138.2 (5.2)			164.1 (14.2)			173.1 (13.1)	

[z](± S.E.)

Table A-7. Wax constituents of 'Dancy' tangerine fruits: Changes in percentage of total wax, quantity per fruit and per unit fruit surface area.

	May 12			June 28			August 10		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	24.0 (4.4)[z]	27.5	9.5	32.1 (4.4)	325.3	12.6	18.0 (0.7)	179.3	3.9
Esters	---	---	---	2.6 (0.2)	26.3	1.0	---	---	---
Ketones	1.0 (0.2)	1.1	0.4	16.0 (1.3)	162.1	6.4	17.5 (5.1)	174.3	3.7
Aldehydes	9.8 (2.0)	11.2	3.8	11.0 (2.2)	111.5	4.4	1.1 (0.3)	10.9	0.2
Secondary alcohols	15.3 (1.8)	17.5	6.0	10.5 (1.0)	106.4	4.2	6.9 (2.0)	68.7	1.5
Triterpenyl acetate	---	---	---	0.7 (0.2)	7.1	0.3	---	---	---
Primary alcohols	28.4 (9.2)	32.5	11.2	15.2 (4.1)	154.0	6.0	6.7 (2.8)	66.7	1.4
Fatty acids	6.1 (1.3)	6.9	2.4	8.0 (2.7)	81.1	3.2	37.0 (1.3)	376.6	8.1
Acidic triterpenoid	15.4 (2.1)	17.6	6.0	3.9 (0.4)	39.5	1.5	12.1 (3.9)	120.5	2.6
Means		114.5 (9.6)	36.1 (19.2)		1013.4	39.9		996.3 (257.0)	21.3 (1.4)
Mean surface area (cm²)		2.9 (1.1)			25.4			46.3 (8.9)	

[z](± S.E.).

Table A-7. Continued.

	September 27			November 15			December 15		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	12.7 (1.7)[2]	260.1	4.2	12.0 (0.6)	658.9	5.7	11.6 (4.2)	533.2	5.1
Esters	6.1 (2.2)	124.9	2.0	6.4 (0.8)	351.4	3.0	4.3 (1.4)	197.7	1.9
Ketones	2.8 (3.9)	57.3	0.9	---	---	---	---	---	---
Aldehydes	21.2 (1.4)	434.2	7.0	28.6 (2.6)	1570.5	13.7	39.6 (2.2)	1820.4	17.6
Secondary alcohols	7.6 (4.5)	155.6	2.5	4.5 (0.5)	247.1	2.1	1.6 (1.3)	73.5	0.7
Triterpenyl acetate	3.0 (4.1)	61.4	1.0	4.4 (0.3)	241.6	2.1	3.6 (0.5)	165.5	1.6
Primary alcohols	3.3 (1.3)	67.6	1.1	2.9 (1.2)	159.2	1.4	2.4 (0.6)	110.3	1.0
Fatty acids	32.5 (4.4)	666.6	10.8	25.5 (5.0)	1400.3	12.2	23.9 (5.7)	1098.7	10.6
Acidic triterpenoid	10.8 (3.1)	221.2	3.6	15.7 (2.9)	862.1	7.5	13.0 (1.2)	597.6	5.7
Means		2048.0 (302.9)	33.1 (4.2)		5491.3 (227.5)	47.9 (1.8)		4597.0 (403.7)	44.3 (3.7)
Mean surface area (cm^2)		61.6 (1.4)			114.5 (0.4)			103.6 (0.3)	

[2](± S.E.).

Table A-8. Wax constituents of navel orange fruits: Changes in percentage of total wax, quantity per fruit and per unit fruit surface area.

	May 15			June 8			July 10		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	43.2 (5.1)[z]	554.4	46.9	24.4 (2.1)	305.2	6.8	26.8 (6.6)	844.2	10.8
Esters	---	---	---	---	---	---	---	---	---
Ketones	21.4 (1.7)	274.6	23.2	2.4 (0.1)	30.0	0.7	4.0 (3.2)	126.0	1.6
Aldehydes	1.0 (0.1)	12.8	1.1	26.3 (0.6)	329.0	7.3	25.1 (0.5)	790.6	10.1
Secondary alcohols	12.4 (0.3)	159.1	13.5	0.9 (0.0)	11.2	0.2	4.2 (0.4)	132.3	1.7
Triterpenyl acetate	1.4 (0.1)	17.9	1.5	3.9 (0.0)	48.8	1.1	4.7 (0.2)	148.0	1.9
Primary alcohols	6.4 (1.4)	82.1	6.9	7.2 (4.3)	90.1	2.0	7.7 (0.8)	242.5	3.1
Fatty acids	5.7 (0.2)	65.9	7.3	32.3 (3.0)	404.1	9.0	23.2 (4.8)	730.6	9.4
Acidic triterpenoid	7.5 (1.5)	96.2	8.1	2.6 (0.3)	32.5	0.7	4.3 (0.6)	135.4	1.7
Mean		1283.4 (11.2)	114.4 (37.4)		1251.1	28.0		3150.0	40.5
Mean surface area (cm^2)		11.8 (3.7)			44.7			77.8	

[z](± S.E.).

Table A-8. Continued.

	August 21			October 6			November 20			December 14		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	7.6 (0.3)[z]	677.2	5.0	9.5 (0.1)	1221.4	6.7	16.4 (1.6)	2990.6	15.3	8.7 (0.9)	1428.6	7.9
Esters	0.7 (0.0)	62.4	0.4	1.5 (0.0)	194.4	1.0	0.2 (5.4)	1495.3	7.6	1.1 (0.1)	183.7	1.0
Ketones	---	---	---	---	---	---	---	---	---	---	---	---
Aldehydes	50.7 (2.1)	4517.5	33.6	25.0 (2.4)	3370.2	18.4	30.6 (4.0)	5530.0	28.5	35.3 (1.4)	5793.0	32.0
Secondary alcohols	---	---	---	0.8 (0.0)	103.7	0.5	1.1 (0.1)	200.6	1.0	---	---	---
Tetracosyl acetate	7.8 (0.8)	696.0	5.1	9.6 (0.5)	1244.4	6.8	11.8 (6.5)	2151.7	11.0	8.9 (1.5)	1461.8	8.1
Primary alcohols	11.3 (4.7)	1006.8	7.5	14.3 (0.5)	1853.6	10.1	8.3 (0.1)	1513.5	7.7	9.0 (0.5)	1478.2	8.1
Fatty acids	13.0 (1.8)	1211.8	9.0	24.3 (0.4)	3140.3	17.2	19.2 (4.2)	3501.2	17.9	26.2 (3.7)	4303.3	23.7
Acidic triterpenoid	8.3 (1.1)	739.5	5.5	14.0 (5.1)	1814.7	9.9	4.4 (2.5)	802.3	4.1	10.9 (5.2)	1790.3	9.9
Means		9010.3	66.2		12932.4 (520.9)	71.1 (2.1)		18235.3 (265.2)	93.1 (3.9)		16425.0 (1461.3)	90.0 (8.0)
Mean surface area (cm²)		134.6			182.5 (12.6)			195.6 (1.1)			181.1 (3.0)	

[z](± S.E.).

Table A-9. Wax constituents of 'Bluegem' blueberry leaves: Changes in percentage of total wax, quantity per leaf and per unit leaf area.

	March 11			March 26			April 9		
	% of total wax	µg per leaf	µg/cm^2 unit area	% of total wax	µg per leaf	µg/cm^2 unit area	% of total wax	µg per leaf	µg/cm^2 unit area
Paraffins	10.5 (0.3)[z]	39.7	8.8	7.8 (5.4)	77.6	5.8	3.2 (2.4)	46.8	2.5
Esters	---	---	---	---	---	---	---	---	---
Ketones	---	---	---	---	---	---	---	---	---
β-Diketones	34.2 (7.9)	129.3	28.7	40.9 (2.5)	406.9	30.6	54.3 (2.3)	793.9	42.6
Aldehydes	1.0 (0.2)	3.8	0.8	15.3 (5.1)	152.2	11.4	6.7 (1.2)	97.9	5.2
Secondary alcohols	22.0 (2.9)	83.2	18.5	8.8 (9.6)	87.5	6.6	---	---	---
Triterpenyl acetate	2.2 (1.8)	8.3	1.8	2.0 (2.7)	19.9	1.5	1.3 (0.3)	19.0	1.0
Primary alcohols	10.5 (2.9)	39.7	8.8	10.5 (0.3)	104.4	7.8	10.7 (3.2)	156.4	8.4
Fatty acids	10.0 (2.5)	37.8	8.4	7.3 (0.7)	72.6	5.4	11.6 (0.6)	169.6	9.1
Acidic triterpenoid	9.7 (2.5)	36.7	8.1	7.5 (1.7)	74.6	5.6	12.2 (2.5)	178.4	9.6
Means		378.1	83.2		994.9	74.5		1462.2 (31.4)	78.6 (5.7)
Mean surface area (cm^2)		4.5			13.3			18.6 (1.8)	

[z](± S.E.).

Table A-9. Continued.

	May 15			June 8			July 10		
	% of total wax	µg per leaf	µg/cm^2 unit area	% of total wax	µg per leaf	µg/cm^2 unit area	% of total wax	µg per leaf	µg/cm^2 unit area
Paraffins	5.5 (0.5)[z]	142.7	7.4	8.2 (5.3)	106.7	8.9	8.8 (1.5)	171.1	7.5
Esters	---	---	---	---	---	---	---	---	---
Ketones	---	---	---	2.5 (0.1)	32.5	2.7	2.9 (2.0)	56.4	2.4
β-Diketones	51.1 (4.1)	1325.9	69.4	41.6 (2.9)	541.4	45.1	26.4 (0.6)	513.2	22.4
Aldehydes	3.4 (0.8)	88.2	4.6	2.5 (0.1)	32.5	2.7	1.2 (0.3)	23.3	1.0
Secondary alcohols	---	---	---	---	---	---	---	---	---
Triterpenyl acetate	2.6 (1.6)	67.4	3.5	5.1 (1.8)	66.4	5.5	5.7 (1.0)	110.8	4.8
Primary alcohols	17.6 (0.1)	456.7	23.9	16.0 (2.2)	208.2	17.3	17.8 (1.2)	346.0	15.1
Fatty acids	9.7 (1.5)	251.7	13.1	12.9 (2.2)	167.8	13.9	13.8 (3.9)	268.3	11.7
Acidic triterpenoid	10.1 (4.1)	262.1	13.7	11.2 (1.5)	145.7	12.1	23.4 (2.4)	454.9	19.8
Means		2594.8 (49.3)	135.8 (10.2)		1301.5 (57.3)	109.1 (3.9)		1944.0	84.8
Mean surface area (cm^2)		19.1 (1.1)			12.0 (0.1)			22.9	

[z](± S.E.).

Table A-9. Continued.

	August 21			October 6			November 20		
	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area	% of total wax	µg per leaf	µg/cm² unit area
Paraffins	3.8 (1.8)[2]	56.8	2.4	2.0 (0.0)	27.6	1.3	1.8 (1.3)	22.6	1.2
Esters	---	---	---	1.0 (0.2)	13.8	0.6	0.6 (0.4)	7.5	0.4
Ketones	---	---	---	---	---	---	---	---	---
β-Diketones	9.8 (1.0)	146.5	6.2	9.7 (0.5)	133.9	6.3	8.6 (0.2)	108.0	5.9
Aldehydes	6.3 (0.3)	94.2	3.9	2.4 (0.1)	33.1	1.5	7.9 (1.0)	99.2	5.4
Secondary alcohols	---	---	---	---	---	---	---	---	---
Triterpenyl acetate	0.6 (0.1)	8.9	0.4	0.8 (0.5)	11.0	0.5	---	---	---
Primary alcohols	24.2 (0.0)	361.8	15.2	31.3 (4.3)	432.3	20.4	23.7 (4.0)	297.6	16.4
Fatty acids	11.8 (6.2)	176.4	7.4	12.3 (6.3)	169.9	8.0	10.0 (2.6)	125.6	6.9
Acidic triterpenoid	43.5 (6.6)	650.4	27.4	40.5 (2.2)	559.4	26.4	47.4 (8.7)	595.2	32.9
Means		1495.1	62.9		1381.3	65.0		1255.7 (55.6)	74.7 (2.7)
Mean surface area (cm²)		23.7			21.2			18.1 (1.9)	

[2](± S.E.).

Table A-10. Wax constituents of 'Bluegem' blueberry fruits: Changes in percentage of total wax, quantity per fruit and per unit fruit surface area.

	April 9			April 24			May 15			June 5		
	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area	% of total wax	µg per fruit	µg/cm² unit area
Paraffins	5.5	18.7	8.9	1.7 (0.1)[1]	11.3	4.7	0.8 (0.0)	8.2	2.2	7.0 (0.1)	130.6	20.8
Esters	---	---	---	---	---	---	---	---	---	5.1 (1.6)	95.4	15.1
Ketones	---	---	---	---	---	---	---	---	---	---	---	---
β-Diketones	56.2	190.7	90.8	49.9 (3.8)	332.0	138.3	61.7 (5.5)	630.8	170.4	48.6 (6.4)	903.3	144.3
Aldehydes	6.2	21.0	10.0	5.7 (2.9)	37.9	15.8	---	---	---	2.6 (0.8)	48.5	7.7
Secondary alcohols	---	---	---	0.7 (0.3)	4.6	1.9	2.6 (1.4)	26.6	7.2	0.8 (0.3)	14.9	2.3
Triterpenyl acetate	3.7	12.5	5.9	4.8 (4.8)	31.9	13.3	0.7 (0.4)	7.1	1.9	2.3 (0.1)	43.0	6.8
Primary alcohols	3.7	12.5	5.9	7.7 (0.7)	51.2	21.3	0.4 (1.9)	85.9	23.2	13.1 (0.7)	245.1	39.0
Fatty acids	6.4	21.7	10.3	9.3 (2.9)	61.8	25.7	3.7 (0.6)	37.8	10.2	9.1 (1.2)	170.2	27.0
Acidic Triterpenoid	18.3	62.1	29.5	20.2 (0.3)	134.4	56.0	22.1 (2.4)	225.9	61.0	11.4 (6.3)	213.3	33.8
Means		339.4	168.8		665.3	290.9		1022.4	273.3		1871.0	295.2
Mean surface area (cm²)		2.1			2.4			3.7			6.3	

[1](± S.E.).

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He is married to the former Claire Reynolds and they have two children, Kirsty Anne and David Scott.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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